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TITLE: Non-Immunogenic Structrually and Biologically Intact Tissue Matrix Grafts for the Immediate Repair of Ballistic-Induced Vascular and Nerve Tissue Injury in Combat Casualty Care

PRINCIPAL INVESTIGATOR: Nathaniel Bachrach, Ph.D.

CONTRACTING ORGANIZATION: LifeCell Corporation
Branchburg, New Jersey 08876

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Nathaniel Bachrach, Ph.D.

E-mail: nbachrach@lifecell.com

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14. ABSTRACT

Purpose: Produce decellularized and freeze-dried umbilical vessel grafts to serve as off-the-shelf vascular graft prostheses for below-the-knee vessel replacement, AV access, and other clinically relevant applications.

Procedures for procurement, dissection, and processing of human umbilical veins, including decellularization and cryo or solution preservation, were optimized. The umbilical artery was found unsuitable for use. Veins were successfully processed with rhDNase instead of bovine DNase, and gamma irradiated to reduce the risk of disease transmission. Processed veins were found to maintain matrix structural integrity, biomechanical properties and were biocompatible and biologically active. Umbilical vein grafts were evaluated acutely *in vivo* as arterio-venous shunts in both pigs and dogs and were easily accessed with needle despite their anatomical coiling. When grafts were implanted in the same porcine model for a 12-day period, they remained palpable and 100% patent under high flow conditions. In addition, the lumen re-endothelialized in central areas of the grafts despite a significant cross-species inflammatory response. To limit cross-species immune issues a non-human primate model was developed and used to demonstrate patency and healing of the vein graft up to 6 weeks as an arterio-venous shunt.

15. SUBJECT TERMS
Vascular graft, freeze-dried, umbilical, biological activity, repopulation, patency, arterio-venous shunt, nerve graft

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I. Introduction:

It was anticipated that umbilical vein and artery grafts implanted clinically would demonstrate patency as well as significant repopulation and remodeling by the surrounding host tissue. Such a graft would be a desirable alternative to current interventions for hemodialysis access; occluding non-remodeling synthetic PTFE grafts and inconsistent native fistulas, below-the-knee vessel replacement; amputation, and coronary artery bypass grafting.

During the period of this grant the production of decellularized and freeze-dried vascular grafts from human umbilical veins was accomplished while substantially maintaining tissue microstructure biological activity and biomechanical properties. Further research demonstrated the patency and biological response to the graft in a primate model. The development of human umbilical arteries was not successful.

With the goal of an off the shelf graft for combat care different methods for both preservation and shipment of the grafts were developed. As adverse events have occurred clinically for human tissue products acceptable sterilization procedures were also developed.

The results of this research are summarized below under the appropriate objective heading. Detailed reports are referenced and provided as appendices.

II. Objective 1: Optimize procurement and processing protocols for umbilical vessels to obtain acellular grafts that retain matrix integrity and biological activity.

Procurement:

LifeCell Corporation attempted to access umbilical cord tissue from a variety of different types of agencies, including umbilical cord stem cell programs, tissue banks, and hospital obstetrical units. Requirements included procurement of cords within 1 hour of birth and immediate placement in RPMI solution with 300 mg/L L-glutamine and 10 mg/L gentamycin sulfate. Umbilical cords were to be stored at 4°C until time of shipment and maintained on wet ice during shipment. Tissue would be received at LifeCell within 24 hours of birth and would not contain any holes, tears, or clamp marks. Availability of serological test results for safety purposes would be desirable but only necessary as the project moved into the clinical phase.

Securing a tissue source for Research

National Disease Research Interchange (NDRI), located in Philadelphia, is a contract tissue bank organization which provides a host of human tissues for research purposes. Funded by the United States government, NDRI also provides tissue at a discounted rate for organizations funded by government grants. In April 2002 LifeCell's application to receive human umbilical cord for the purposes of producing a non-immunogenic structurally and biologically intact vascular graft was approved.

The arrangement with NDRI was very positive, with LifeCell receiving intact umbilical cords in response to the majority of our requests. However, NDRI has shown to be limited in resources both pertaining to tissue sources and increased umbilical cord availability as well as patient serology. LifeCell received umbilical cords from NDRI for preclinical evaluation and research.

Viable Procurement Procedure for Human Tissues for Clinical Use

LifeCell Corporation has demonstrated a viable procurement model for the collection of human umbilical tissue. A procurement arrangement was successfully implemented with Saint Francis Hospital, Department of Labor and Delivery, located in Tulsa, Oaklahoma. The SFH internal review and ethics board (IREB) has approved a clinical procurement protocol and informed consent for obtaining the umbilical tissue. The donor is deidentified from LifeCell and SFH has obtained a certificate of confidentiality from the NIH to protect the donor patients' privacy. Consent includes the provision for research use as required by tissue banking standards in order to use the tissue for this clinical research project. All nurses and physicians involved in the collection of research data were trained as required by the IREB at SFH. No umbilical cords or other information was obtained from expectant mothers without a signed informed consent form.

The procedure: The nurse or physician obtains patient's consent at time of admission if initial donor criteria are met. The nurse or physician will then complete the patient assessment questionnaire and assign a patient donor number for use in all documentation that will be seen by LifeCell Corporation. The patient assessment questionnaire includes questions regarding medical and social history and is designed to determine evidence of high risk behavior as defined by the Center for Disease Control.

Following delivery, the cord is cut and the placenta is delivered with as little traction as possible. The cord is then clamped proximally in a way that maximizes usable tissue length and cut from the placenta using a scalpel. Blood is then allowed to drain from the cord and the cord is placed in a sterile container with saline. The cord is then transported to the pathology laboratory on ice for detailed assessment to previously defined rejection criteria.

The Umbilical Cord Data Form is then filled out, in part by labor and delivery and in part by the placentologist. This data form documents the informed consent, the cord dimensions, some

basic donor information (mother and child), a physical assessment of the mother for risk factors, and the pathologist's gross inspection of the tissue.

Process Development:

Dissection

Umbilical vessels are very well protected within the umbilical cord. Both arteries and vein are surrounded by a protective sheath of Wharton's jelly which is encased within a tough amnionic membrane. In order to access these vessels for graft processing, both the Wharton's jelly and most of the amnion must be carefully dissected away from the vessels. This task is made difficult by the fact that the vessels themselves are very thin and friable, do not have a true adventitia and are therefore mostly indistinguishable from the surrounding tissues, and tend to spiral to varying degrees.

Scalability is a very important issue, and without a reliable way to produce large quantities of umbilical grafts quickly, processing and testing of grafts would be impossible. Using standard dissection methods the harvesting of the umbilical vein from the cord takes 3-4 hours and is prone to error that can compromise the vessels sought. Much of the difficulty in dissection is caused by the natural coiling of the vessels within the cord and the low visual contrast between the vessels and the surrounding tissue. LifeCell has developed a method for the dissection of umbilical veins and arteries from umbilical cords that greatly increases not only productivity but also quality of the final graft. We have developed a technique for fixing the vessel to a dissection tray in a way that allows easy rotation during dissection to follow the coil of the vein. To address the issue of contrast we have diluted our cryoprotectant solution to a manageable

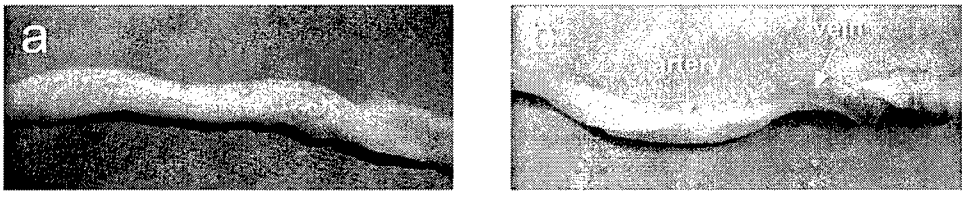


Figure 1. Umbilical cord (a) unperfused and (b) perfused with cryoprotectant.

viscosity and added a diagnostic indicator dye, Evans blue, at a concentration of 6.7 μg/mL. The vein is filled with the solution for visual contrast and pressurized for physical contrast (Figure 1) both helping to decrease the rate of damage. Perfusion of the umbilical vein with dyed cryoprotectant also aids in the removal of umbilical arteries from the cord by providing a darker background against which to visualize them. Using the developed methodology has minimized dissection error and reduced dissection time to <1.5 hours for a 25-30cm umbilical cord.

Holding Step

Since procurement of tissue is variable and graft processing should lend itself to convenience and scalability, a holding step, was introduced into the vascular graft process. Previously, tissues were harvested fresh and processing began immediately. Since infant births are variable, neither the time nor the quantity of umbilical cord availability is predictable. With the addition of a holding step, cords could be collected and frozen until an appropriate lot size has accumulated. Then the lot can be processed at a convenient time.

Currently, umbilical veins and arteries enter the holding step after dissection. The umbilical vein is filled with dyed cryoprotectant, ligated, dissected free from the surrounding tissue, and then submerged and incubated in cryoprotectant solution at 4°C overnight with rotated mixing. Following incubation, the vein and arteries are removed from the surrounding cryoprotectant

solution and frozen at -80°C in new containers. Vessels can then be held at this deep-freeze step for a prolonged period of time prior to processing.

A viable manufacturing process must allow a hold before any value added processing of the umbilical cords to provide time for receipt and review of all donor information and evaluation of presence of pathogens. To achieve this, the holding step described above has been shifted to occur prior to umbilical cord dissection. In the new process the umbilical vein is filled with cryoprotectant and ligated as before. The entire cord is then submerged in cryoprotectant and incubated overnight at 4°C with rotation and frozen the next day. This process change could only be achieved after determination of cryoprotectant penetration into the tissues through the thick amnionic membrane and layer of Wharton's Jelly. A transmission electron microscopy study has shown that compared with fresh tissue, the tissue processed after the whole cord holding step retains the basic essential microstructural elements of the tissue while eliminating the potential for cell mediated responses to the tissue when implanted (Appendix A). A study was also performed to evaluate the thermal properties of the tissue as a measure of effective cryoprotectatnt incubation (Appendix B). The study concluded that the vessels in the umbilical cord are cryoprotected to the same extent as dissected tissue processed by previously developed methods.

DNA removal

Previous work to produce decellularized vascular tissue by LifeCell involved the use of bovine DNase I at a concentration of 150 Kunitz units/mL solution. This step allowed for the enzymatic digestion of residual nuclear remnants following solubilization of cell plasma and organelle membranes with n-octylglucopyranoside detergent treatment. Enzymatic digestion was performed at the optimal temperature for the enzyme, 37°C.

In an effort to render our human umbilical vascular graft as safe as possible, two processing parameters were changed pertaining to the nuclear enzymatic digestion. Due to the risk of contracting Creutzfeldt-Jakob disease from bovine spongiform encephalopathy contamination, we changed our nuclease to a human recombinant deoxyribonuclease. Marketed by Genzyme under the name Pulmozyme, this rhDNase is clinically used to treat cystic fibrosis. Additionally, although 37°C is the optimal temperature for Pulmozyme activity just as it is for bovine DNase I, it is also the optimal temperature for the growth of bacterial cultures. Although an antibiotic cocktail is used in both the detergent and enzyme steps of the processing protocol as a prophylactic agent, incubation of the harvested tissue at 37°C to optimize nuclease activity could actually promote bacterial growth in the system.

The effectiveness of Pulmozyme at various concentrations, temperatures, and lengths of incubation was evaluated. Histological evaluation of cell removal and biochemical quantification of residual DNA led to an optimal processing protocol (Appendix C). The final process includes Pulmozyme at 40 μg/mL (for both umbilical arteries and veins) at a tissue weight to solution volume ratio of at least 20 mg tissue/mL enzyme solution for umbilical arteries, and 40 mg/mL for umbilical veins. Vessels treated with these conditions were consistently found to be decellularized.

Biocompatibility

Processing protocols have been designed to achieve biocompatible vessels that would be acceptable for the intended use - clinical implantation. The umbilical artery process has not yet been optimized (see section on artery processing) so only the umbilical vein graft (UVG) has been tested to date. Table 1 provides summarized the biocompatibility testing that has been competed on the UVG. A more detailed review of the tests and their results is provided in Appendix D.

Table 1: Summary of biocompatibility tests completed testing for the UVG

Test	Test Method	Test Article	Result
Cytotoxicity	Test system: in vitro L929 mouse fibroblasts, 48hr @ 37°C • Elution: 2gm tissue @ 37°C for 24 hr in 10ml applied to test system • Agar Diffusion: lumenal surface applied to 0.5-2g/ml agar layer on test system	Frozen UVG	Not cytotoxic
Hemolysis	Test system: human red blood cell concentrate • Direct contact:0.1gm applied directly to test system • Extracted:0.1g tissue @ 37°C for 24 hr in 1.5ml saline applied directly to test system	Frozen UVG	Not hemolytic
Pyrogenicity	Test system: White rabbits' febrile response • Extraction: 4g tissue @ 37°C for 24 hr in 80ml saline	Frozen UVG	Not pyrogenic

Quality Plan

As a fundamental part of processing optimization in the development of a graft that will have use in combat casualty care it is essential that it be developed with a robust quality plan. Part of the necessity of this plan is a quality assurance level even on development grafts used for in vitro and in vivo testing that will be reliable. This is critical in light of the graft related events that have been found in those in vivo studies and uncertainty regarding their processing environment.

Procedures have been developed to minimize the chance of infection during UVG processing consistent with general tissue industry practices such as donor screening, microbiological testing, antibiotic and antifungal treatments, utilizing sterile processing supplies, and a controlled processing environment. The processing and storage of the tissue at the LifeCell facility conforms to 21 CFR 1270 Human Tissue Intended for Transplantation and the American Association of Tissue Banks (AATB) Standards for Tissue Banking. The details of what is included in the plan are provided in Appendix E.

Cold-Gamma Irradiation of Umbilical Tissue

Before and/or After Processing

A study was conducted to evaluate the potential to increase the safety profile of the human umbilical vein graft (UVG) through gamma irradiation (Appendix F). Since gamma irradiation is known to affect the structural and biological properties of tissues a cold gamma scheme was developed to try and avoid some of the damaging effects of the radiation. An initial study was designed to determine the effect of different γ-irradiation schemes on the following: processing (i.e. difficulty of dissection, pliability, etc), X-101 penetration, histology, electron microscopy, suture retention, circumferential tensile compliance, and test to failure.

Seven umbilical veins were each divided into the following conditions:
W: Control, no γ-irradiation

X: pre-dose of 12 kGy γ-irradiation
Y: final dose of 12 kGy γ-irradiation
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation
Samples were packaged for γ-irradiation as per the protocol and stored at approximately -80°C until the time of testing. The in vitro characterization data from both the mechanical testing (Table 2) and the histological analysis (data not shown here) show that no significant differences between any of the treatments.

Table 2: Mechanical Testing Data (n=5; avg + std dev)

Treatment	Suture Retention Strength (N)	Compliance (N/%strain)[1]	Break (N)
W: Control, no γ-irradiation	4.39 ± 1.91	6.08 ± 1.58	18.95 ± 7.49
X: pre-dose of 12 kGy γ-irradiation	4.28 ± 0.80	9.21 ± 5.08	15.83 ± 5.66
Y: final dose of 12 kGy γ-irradiation	4.95 ± 3.06	7.56 ± 3.48	16.66 ± 6.76
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation	4.25 ± 0.90	8.94 ± 8.99	16.05 ± 4.07

Reduced rehydration was observed in the post-dose (Y) and double-dosed (Z) conditions (Table 3). This appears to verify a visual observation that the Wharton's Jelly appeared shrunken and collapsed.

Table 3: Rehydration Data

Treatments	Packaging method before rehydration analysis	Hydration after γ treatment (g water per g dry tissue) *	Hydration after full rehydration (g water per g dry tissue)
W	Tyvek/Foil bag	15.6 ± 1.9	25.0 ± 1. 9
X	Tyvek/Foil bag	12.6 ± 0.9	16.7 ± 1.2
Y	Tyvek/Cassette	19.0 ± 1.6	16.7 ± 0.7
Z	Tyvek/Cassette	13.3 ± 0.3	14.5 ± 1.1

* Data represent the mean ± standard deviation of four measurements (two samples from each of two lots)

This study provided some clues about the effect of γ-irradiation on the umbilical vein graft. However, it is difficult to judge which treatment scheme can be considered optimal. Also, as the level of damage to the tissue seemed negligible a follow-up study in which higher γ-irradiation dosages are utilized was pursued.

[1] For the compliance test, three samples were tested from each graft. The average of the three measurements for each of the five grafts was then used to generate the data reported in Table 2.

In Vitro Dosing Evaluation

A method for maintaining cold (-80°C) temperatures during γ-irradiation was shown to provide a degree of protection of the tissue from damage. A study was undertaken to determine the dose response of a cryoprotected and frozen graft to γ-irradiation at different dosages (0 – control, 12, 18, and 24 kGy). It has also been found that the damaging effects of gamma irradiation can some times be seen in terms of the stability of the tissue with changes occurring over time. Therefore samples were also evaluated for the effect of ambient temperature storage (30 days) on non-cryoprotected grafts that had been γ-irradiated at 12 kGy. The storage solution can also play a role as a gamma protectant so an additional objective was to determine the equivalence of ambient temperature stored, non-cryoprotected grafts to cryoprotected grafts stored at -80°C (eg storage solution). After irradiation the grafts were evaluated for qualitative changes (color, handling etc), histological changes in the tissue's structure and the mechanical properties of the vessel. A complete description of the study and the results are presented in Appendix G. The conclusions are summarized here.

Gamma irradiation appeared to have the most effect on tissue handling characteristics. Although γ-irradiated and ambient stored cryoprotected grafts became yellower and darker in color after 30 days, the color change did not appear to have an effect on the mechanical testing results. Histological analysis showed that storage at -80°C in PBS for both γ-irradiated and non-irradiated samples produced more damage to the collagen fibers and created holes in the Wharton's Jelly. The γ-irradiation dosage does not affect the mechanical properties of cryoprotected grafts at time 0.

The use of PBS (ambient storage) did not produce significant changes in mechanical properties compared to cryoprotected grafts at time 0. Ambient storage conditions for cryoprotected grafts did not appear to affect the mechanical properties. Both storage time and γ-irradiation at 12 kGy, affected the mechanical properties of non-cryoprotected samples – ie γ -irradiation reduced compliance by 55%, storage time reduced load on break by 24%, and there was an interaction in which suture strength dropped by 66% with storage time for non-irradiated samples.

Matrix integrity

Lumenal defects in the freeze-dried umbilical vein grafts

In the study that was used to evaluate the porcine AV-shunt system for use as a chronic implant model it was found that one graft had significant hemorrhagic dissection of the media. These findings suggest that the structural integrity of the umbilical vein graft (UVG) had been compromised. The root cause of this breech in structural integrity was investigated and two distinct causes were found to have contributed to this failure:

- Defects were detected on the lumenal surface of that explanted graft. Gross in vitro evaluation of the lumenal surface of freeze dried UVGs as produced for this experiment revealed occasional crevices. This damage has been demonstrated to result from the particular freeze-drying process used, and is a directly assignable cause to this mode of failure in the animal model.

- Histopathology of the explanted grafts revealed a cross-species immune response. This kind of response typically includes rapid resorption of extracellular matrix components responsible for a blood vessel's mechanical integrity. Therefore, an accelerated loss of structural integrity is a predictable consequence of implanting intact human tissue in an animal model.

Frozen Configuration

A series of experiments have been performed that demonstrate the source of the cracking of the tissue is the final stages of the freeze-drying process. Currently the only way to provide 100% assurance that vessels do not contain lumenal cracks is to provide a frozen final graft that is thawed at the point of use.

Storing the graft frozen instead of freeze drying represents a significant process change. Firstly the graft had not been frozen below -35°C in the freeze-drying process and now the graft would be held at -80°C. Secondly, the new thawing process without the vacuum provides a different environment for the tissue and has the potential to cause tissue damage. Verification testing was performed on the graft including burst testing, suture retention, and multipuncture mechanics (which is relevant for hemodialysis grafts and can provide hints of more subtle tissue damage for other grafts as well). A full description of the biomechanical testing and failure analysis that were performed is attached as Appendix H.

Other Strategies to Alleviate Tissue Damage

While these results demonstrate that the frozen-thawed UVG is an acceptable graft it is not the optimal process for combat casualty care as controlled storage and transport of the graft at -80°C is not a simple matter.

The most straightforward processing option to avoid luminal defects is to provide a freeze-dried graft that is not dried to the level where cracks are initiated in the tissue. This solution would be viable only if the threshold residual moisture level in the tissue where cracks initiate could be identified. Then adjacent (or satellite) samples of tissue would be evaluated as a quality measure to ascertain that the drying process did not go to far for any given umbilical vein (given the biological variation between donor tissues). Exploring this design option required the following:

1. Development of a robust and accurate method for determining residual moisture levels in umbilical tissue samples
2. Evaluation of the within graft residual moisture variation to determine the limitations on residual moisture for an adjeacent section of tissue.
3. Determination that the satellite sample was indeed representative of the residual moisture in the whole vein segment.
4. Determine the nominal residual moisture threshold where lumenal cracks begin to form in the tissue.

A thermogravimetric method has been developed for determining residual moisture levels in umbilical tissue samples. This method was then applied to evaluate the within and between graft variability, and to compare the satellite samples to those values. It turns out that the variability within a graft is higher than the variability between donor vein grafts. Also, it was determined that satellite samples do not provide a representative measure of the residual moisture in the whole vein segment. These results led to the following conclusions.

1. Freeze-drying to a higher residual moisture level is not an option as there is no way at this time (without 100% destructive testing) to achieve a sufficient assurance level that any given vessel does contain a segment that was dried too much and has developed a lumenal crack.
2. This level of variability in the drying of the tissue is an indication that the drying process has not been sufficiently optimized for this particular complex tissue.

These conclusions led to a series of experiments designed to provide uniform drying of the tissue. All efforts in this area were unsuccessful. In addition, while freeze-dried grafts provide long shelf lives and not very restrictive storage conditions the rehydration of the vessels can take over an hour. Therefore, solution preservation methods were pursued.

Solution Preservation

Development of Preservation Methods

The preservation of umbilical vein grafts is currently conducted with X-101 that allows freezing the grafts to -80°C for storage. In addition to the -80°C storage, considerations are being made toward using a solution capable of preservation at either room temperature or under minimally refrigerated condition (e.g. 4°C). This is a critical design modification as the objective of this work is to develop an off the shelf graft more suitable for combat casualty care. The two choices evaluated thus far are the use of Glycerol (Gly) and Ethylene Glycol (EG) to substitute water in the tissue matrix. The first step in evaluating these components was to develop a process through which systematic dehydration could occur without damaging the tissue matrix and upon washing in saline, complete rehydration could follow. A number of experiments were performed to develop a process for each of the solutions. The data on the ultimately suggested processes are presented in Appendix I.

For both glycerol and ethylene glycol, each equilibration step can be standardized to a processing time of 1 hour ± 15 minutes. The glycerolization process requires a series of 4 increasing concentrations of 40%, 55%, 70%, and finally 85% while ethylene glycol only requires 2 concentrations of 50% and 90%.
After stagnate rehydration of 40 minutes in 10ml saline/cm on the bench top, samples contained ~18% v/v residual glycerol or ~24% v/v ethylene glycol residual. Finally, the amounts of residual moisture in the samples were 12.2% w/w for glycerol and 10.3% w/w for ethylene glycol.

These studies show that glycerol and ethylene glycol are both capable of water replacement for preservation of Umbilical Vein Grafts. These results led to studies to evaluate the effect that these solutions have on the tissue matrix before introduction into the umbilical vein graft manufacturing process. This information would also provide guidance on which solution would be preferable.

Comparison of Preservation Methods In Vitro

An evaluation was performed to compare the two new preservation methods to the current method of frozen preservation in a cryoprotectant. Three cords were dissected then divided in 3 equal pieces of ~13-15 cm each and processed separately until the preservation step as per standard operating procedures (SOP). Each piece was subjected to one of the following three treatments.

- X-101 treatment followed SOP with one 4 hour ± 10 minute incubation (~4 hour) followed by a second 4 to 24 hour incubation (~19 hours)
- Glycerol treatment consisted of a series of 4 incubations with increasing concentrations of 40%, 55%, 70%, and 85% glycerol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rpm.
- Ethylene Glycol treatment consisted of a series of 2 incubations with increasing concentrations of 50% and 90% ethylene glycol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rmp.

Visual observations and measurable characteristics demonstrated that the different treatments do seem to have varying effects on the tissue; however, tissue to tissue variability was also observed. Even though the three treatments leave the tissue with a slightly different appearance, upon re-hydration they all look the same.

Histologically, X-101, glycerol and ethylene glycol had similar evaluations for holes, collagen damage, and collagen separation / orientation for all three sample lots and treatments appeared similar. Also for all sample lots and treatments the basement membrane and internal elastic lamina was present. Lastly no MHC I or II was detected on any of the slides. The only mechanical difference detected between the different treatments was the reduced compliance (N/%) in the Glycerol preserved compared with the X-101 frozen tissue.

Based on the comparison criteria of histology, circumferential, suture retention and burst strength testing the data show that the different processing methods are comparable. The full study report is provided as Appendix J.

Stability evaluation

A glycerol preservation shelf life study was initiated to evaluate the characteristics listed above with an additional test of load at break (N) after multi-puncturing over time. Samples were tested at ~0, 1, and 3 months and were stored at either 4°C or 20°C. The results indicate that histologically over 3 months the samples nay be seeing a change with increased collagen damage orientation and separation. Histological evaluation is a highly variable and the histological methods have not been validated for working with tissue that may contain residual levels of glycerol so this data is not yet conclusive. Mechanically, on the other hand, the data showed tensile strength (load at break and compliance), multi-puncture and the burst strength remained constant over time. The full study report is provided in Appendix K.

Biological Activity

A number of studies have been performed during this research demonstrate that the biological activity is retained by the matrix of the vessel after processing.

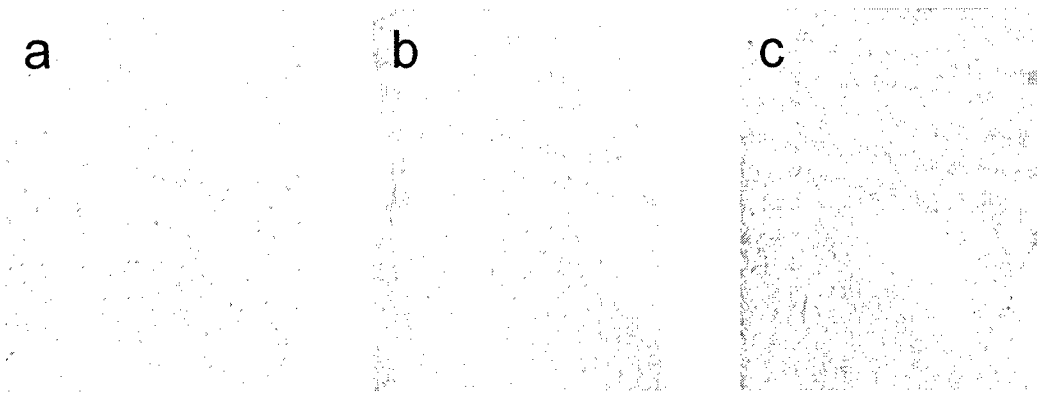
Initial proof of principal in small animal implant study:

Umbilical veins and arteries processed using both Pulmozyme (40 μg/mL) at room temperature and bovine DNase I at 37°C were compared with adult porcine carotid arteries (processed with bovine DNase I at 37°C) and jugular veins (processed with Pulmozyme at room temperature) in a subdermal implant study in both immuno-competent rats and nude mice. Processed samples were freeze-dried and rehydrated just prior to subcutaneous implantation. Samples were explanted from rats at 7 days for evaluation of an acute response and explanted from nude mice at 21 days for evaluation of a chronic response. Biopsies were fixed in formalin, thin sectioned, and stained with hematoxylin and eosin for histological analysis.

Samples were assessed for each of the following on a scale of 0-4:

- Extent of fibrous encapsulation of the graft material by host granulation tissue.
- Extent of inflammation
- Extent and distance of host fibroblast infiltration,and extent of revascularization of the graft material.

Assessment of each of these variables *in vivo* gives a good indication of matrix integrity and biological activity of the processed and implanted samples. Each slide was assigned a value (0-4) for each variable and the values totaled to yield an overall score. High scores indicate overall poor integration of graft and host tissue, while lower scores indicate overall good integration. Results of the nude mouse study are shown below in representative histological sections.



Representative histological sections of biopsies explanted from nude mice following 21 days implantation . Each of these samples was processed with Pulmozyme. (a) umbilical vein, (b) umbilical artery, (c) porcine jugular vein.

All of the histological scores were relatively low and indicative of tissue that is capable of being infiltrated, revascularized and remodeled by host cells in vivo (see Appendix L – Summary Report).

The analysis of the effect of cellular removal process on the different umbilical vessels revealed a number of important results. The process using Pulmozyme resulted in tissue that was significantly more revascularized, less encapsulated and more homogeneously infiltrated. Also, the umbilical vein incites less inflammation, allows more infiltration, and is less encapsulated compared to the umbilical artery.

These results lead to the obvious conclusion that shifting from bDNase to Pulmozyme has a positive effect on the matrix. This is possibly due to more effective removal of the DNA.

The comparisons of the umbilical vessels to the adult artery indicate that when the vein graft is used clinically as an allograft it could perform as well or better than processed ovine arteries did when they were used as allografts with respect to host cell repopulation, vascularization and lack of inflammation. The artery indicated slightly higher inflammation in the rat but still scored in the midrange of the scale and would be considered an acceptable response in this study.

In vivo evaluation after gamma irradiated vein grafts

After promising results with cold gamma demonstrated that there is minimal damage to the tissue in the in vitro evaluations of the tissue a study was undertaken to evaluate the biological response to these tissues. A nude rat implant model was used to assess the biocompatibility of human umbilical vein grafts (UVG) processed under different conditions. Each rat received four subcutaneous implants: a positive control (X-101, frozen UVG), a negative control (ePTFE), and two of six test conditions. The six test conditions were frozen X-101 grafts irradiated at either 12, 18, or 24 kGy post-processing; frozen X-101 irradiated both pre- and post-processing for a cumulative 24 kGys dosage; ambient PBS stored grafts irradiated post-processing at 12 kGys; and non-irradiated freeze-dried grafts. Each test condition was implanted in five rats.

All rats were euthanized after 21 days' implantation. The explants were each photographed, fixed, and stained for histophathological analysis. The overall finding was that there was a similar response to the test articles compared to the positive control as measured by

encapsulation, fibroblast infiltration, neovascularization, and mononuclear cell infiltrates. A quantitative ranking of the conditions yielded relative trends. Compared to the positive control, the single and cumulative dose of 24 kGy conditions were slightly more biocompatible by this scoring methodology while the freeze-dried, PBS, and 18 kGy conditions were less biocompatible. The full study report is on file at LifeCell Corporation (LCP2004-04-03).

This result probably reflects the loss of tissue stability with gamma irradiation that allows faster cell infiltration. This raises the possibility that the tissue that is irradiated beyond 18kGy is damage in a way that could be detrimental in the sense that accelerated remodeling can be catastrophic. A functional tissue substitute, like the UVG, that is expected to regenerate and remodel into host tissue is required to maintain its functional mechanical properties during that transition.

Comparison of Preservation Methods In Vivo

The objective of the study was to assess the biocompatibility of tissues preserved in glycerol or ethylene glycol relative to the current cryopreservation method. In addition both 4 degree and 37 degree storage of the samples was tested (Appendix M). The higher temperature storage would provide an early evaluation of an accelerated storage response. Biocompatibility was determined as well integrated tissue that is populated by non-inflammatory host cells and supported by neovascularization of the tissue following implant. Based on this criteria, the test articles and control samples were ranked from most biocompatible (a score of 1) to least biocompatible (a score of 4) by the pathologist. The various test articles are shown in the table below.

Sample	Tissue Type	Storage Conditions
A	Positive control article	-65° to - 90°
B	Negative control article	ambient
C	Test Article – glycerol	4°C ± 1°C
D	Test Article – glycerol	37°C ± 1°C
E	Test Article – ethylene glycol	4°C ± 1°C
F	Test Article – ethylene glycol	37°C ± 1°C

The data showed that the morphology of the host tissue response was similar for the tissue stored in Glycerol and Ethylene Glycol compared to the cryopreserved tisse. The vein grafts were infiltrated by fibroblasts and new blood vessels indicating a favorable biocompatible response and the potential for host cell regeneration and homeostasis of the tissue. The degree of response varied as is to be expected in this type of qualitative evaluation. An attempt was made to assign biocompatibility scores to the samples and then compare in a semi-quantitative analysis. These evaluations were made by the pathologist and are presented along with a study report in Appendix M. The intensities of alterations varied somewhat between each test article as well as between some test articles and the positive control umbilical vein grafts. Test articles stored at 4° C gave similar response to the positive control, while test articles stored at 37° C had less biocompatibility compared to the positive control with a slight increase in the fibroblast infiltration and neovascularization. It was concluded from this study that the acute biocompatibility of the UVG stored in glycerol or ethylene glycol is acceptable. More studies are necessary to fully evaluate the stability of the tissue over time at different temperatures.

III. Objective 2: Establish the biomechanical characteristics of these unique, biologically intact umbilical vessel grafts in failure mode analysis.

Biomechanical testing is addressed in Appendix H referenced in Objective 1 (above) as part of Matrix Integrity.

IV. Objective 3: Demonstrate that umbilical vessel grafts transplanted, as carotid interposition implants, in an animal model system maintain patency in the absence of dilatation, aneurysm formation or neointimal hyperplasia.

Umbilical arteries were implanted in a porcine model as carotid interposition grafts. The arteries used in that experiment were frozen-thawed grafts to avoid the lumenal cracking that had been discovered with the vein graft (see matrix integrity in Objective 1). These grafts were implanted for a 7 day period to allow for relevant data to be collected without risking an immune response severe enough to compromise graft function. The grafts failed due to rupture of some kind and the animals did not survive the experiment. The details of the experiment are presented in Appendix N. It is not certain what cause the fate of these implants. However, a exhaustive evaluation of the ability to obtain substantial intact and robust sections of umbilical arteries have been performed to try and determine if the root cause of the failures lies in an inherent weakness of the tissue or one introduced during dissection. Shredding and weak areas of the arteries has been discovered in areas where gross observation does not indicate such damage (Appendix O). While efforts were made to optimize processing for the artery to provide robust tissue for implantation the conclusion from that work is that at this time the artery from the umbilical cord is not a viable source for vascular tissue for implantable grafts.

V. Objective 4: Demonstrate in an animal model that these umbilical vessel matrix grafts maintain patency in clinically relevant application simulating long length with low flow dynamics.

Relatively long processed umbilical vein grafts were preliminarily assessed as a high to low flow shunt in both porcine and canine acute models and in a porcine 12-day model. This model was chosen to determine the ability of the graft to conduct flow between high and low pressure systems, giving some indication not only of graft stability and resistance to aneurysmal dilatation near the arterial anastomosis, but also resistance to low flow induced stagnation near the venous anastomosis. This model also allowed for the implantation of two grafts within one animal without serious risk of one graft, or the life of the animal, being compromised by potential failure of the other graft. The study reports from those studies are included as Appendix P (porcine) and Appendix Q (canine). After successfully implanting the grafts and maintaining acute patency two grafts were implanted for twelve days in the porcine model to see if the model would be suitable for longer term implants to evaluate the remodeling of the tissue in vivo. The immediate goal was to assess patency, flow, re-endothelialization, and the extent of cross-species immune response in this model. Both grafts did exhibit a significant inflammatory response potentially due to cross species implant, which was evident by histological analysis. The study report is included as Appendix R. This kind of response to the tissue is a strong indication that the matrix components are well preserved as per the objective of the project and motivated the development of a primate model to provide compelling data regarding the clinical utility of the graft. A primate model was developed to try and avoid cross-species response to the grafts and provide longer term data on the repopulation of the umbilical vein graft and its potential to remain patent in vivo.

Human Umbilical Vein Grafts were implanted in large male vervets (one per animal) as an AV shunt from the Iliac/femoral artery to the femoral vein. Needle access was planned as a possible endpoint to demonstrate the potential for a chronic multi-stick model. Grafts were implanted for 2 and 6 weeks, three for each time point, with 3 additional grafts included with an unplanned duration. Synthetic control grafts were also implanted (one graft for each time point). Serum was obtained prior to and during the study to evaluate the immune response of the animal to the graft. The study demonstrated that the umbilical vein graft (UVG) can be implanted as an arteriovenous shunt from the iliac artery to the femoral vein in this primate and remain patent for as long as six weeks. The data showed that there is a humoral immune response to the graft in this model. However, despite this response, no mural degeneration or aneurysms in the graft were noted within the first six weeks. An initial inflammatory reaction to

the graft was shown to resolve over time giving way to a healing response and the initiation of new tissue growth. Taken together these results indicate that although this is a xenogeneic model (Human to Monkey), it may provide a means of obtaining information about the expected function, hemocompatability and healing response of the UVG for as long as six weeks in vivo. This study also demonstrated that the graft can be cannulated in the chosen position and that hemostasis after cannulation of the UVG compares favorably to ePTFE. No grafts were available to extend beyond the six week timepoint due the disruption of the grafts by the animals. The study report is attached (Appendix S).

Based on the success of the animal model an additional study was designed to obtain chronic patency data. The initial implant phase of the study was successful. However, there were a series of occlusions noted early in the study and evaluation of the grafts indicated that there had been surgical complications that had led to the occlusion of a number of grafts. After a further evaluation of the remaining implants indicated that more grafts were becoming occluded the study was terminated. The cause of occlusion in these grafts has been investigated however no definite conclusions could be drawn. There were differences in the second study with respect to surgical procedure including placement of the graft to a deeper and more proximal location. Also, a significant amount of suturing and closure tension was applied to keep the wounds closed and protect the grafts from the animals. Both factors are likely to have contributed to the occlusions. In addition the study was designed to evaluate the healing of the graft with cannulation and this had not been part of the model development.

Although the second study raises significant questions about the suitability of the model for AV access the results of the first study clearly indicate the potential for this graft to remodel and remain patent long term as an allograft for human use.

VI. Objective 5: Optimize procurement and processing protocols for nerve tissue to obtain an acellular graft that retains matrix integrity and biological activity.

Preliminary work done with nerves has included harvesting of sciatic, median, radial, and ulnar nerves from 6 rabbits at Montefiore Medical Center (Bronx, NY) followed by subsequent decellularization processing, cryoprotection, freeze-drying, and micronization.

VII. Objective 6: Demonstrate repopulation of these unique, biologically intact nerve matrix grafts and restoration of neural function in a clinically relevant nerve defect model system.

The intent of the research was to inject the micronized nerves discussed in Objective 5 into nerve guidance channel defect models in rabbits to act as a source of neurotrophic factors to spur nerve regeneration. This research was objective not achieved due to the unanticipated hurdles in the development of the arterial grafts and the necessary modifications to the processing of the vein graft to include, solution preservation, sterilization and the development a nonhuman primate animal model.

VIII. Key Research Accomplishments:

- Procurement and Processing
  - Established a viable procurement model for procurement of human umbilical cords suitable for clinical use.
  - Developed quality plan, including processing controls and tissue evaluation for the controlled processing of umbilical tissues.
  - Instituted a holding step during which umbilical vessel grafts can be cryopreserved until serological data is obtained and a convenient processing time is reached.
  - Developed a reliable method of efficient dissection for umbilical veins from umbilical cords with little to no resulting damage.
  - Validated, in vitro and in vivo, the use recombinant human deoxyribonuclease (Pulmozyme) to replace bovine DNase in decellularization of umbilical veins and arteries.
  - Demonstrated that the processed veins are still microstructurally intact even at the electron microscopy level.
  - Demonstrated biocompatibility of the processed vein grafts.
  - Determined a method for gamma irradiating the umbilical vein for an increased safety profile without causing significant acute damage to the tissue as evidenced by in vitro and acute subdermal implant evaluations.
  - Developed two alternate preservation methods to allow higher temperature storage and shipping for improved functionality.
  - Measured mechanical properties of umbilical arteries and veins before and after processing for multiple graft configurations including freeze-dried, cryopreserved, glycerol and ethylene glycol preserved demonstrating their suitability for clinical use.
  - Established the stability of the vein graft for as long as 3 months.
- Pre-clinical Studies
  - Established the biocompatibility and bioactivity of multiple graft configurations including freeze-dried, cryopreserved, glycerol and ethylene glycol preserved in acute small animal models.
  - Successfully implanted 8 umbilical vein grafts acutely in 2 distinct animal models as arterio-venous shunts under high flow conditions.
  - Implanted umbilical vein grafts that, despite a severe cross-species inflammatory response, remained 100% patent for 12 days and exhibited some re-endothelialization.
  - Developed a primate model to evaluate the response biological response to the graft under arterio-venous flow conditions for up to 6 weeks.
  - Demonstrated in a primate model that the vein graft is capable of being sewn in place and maintaining patency for up to 6 weeks. And that during that time the tissue begins to become remodeled by the animal and is well integrated into the adjacent vessels.

IX. Reportable Outcomes:

N/A

X. Conclusions:

The production of decellularized and freeze-dried vascular grafts from human umbilical vessels was accomplished while substantially maintaining tissue microstructure biological activity and biomechanical properties. The data show that it is possible to preserve this tissue matrix and its bioactivity without compromising its mechanical integrity through the use of water removal methods and molecules like glycerol. It is also clear that some level of Bioburden reduction is possible with this non-sterile human derived graft that can provide additional safety to the patient. As adverse events have occurred clinically with human tissue products it is becoming an essential design aspect for aseptically processed tissues to provide some level of microbial reduction. This is an area of intense research and the ability to provide sterile tissue products with intact matrix bioactivity would be an essential aspect to providing these much needed regenerative tissue products to patients with critical unmet needs.

It has been demonstrated now that the umbilical vein grafts implanted for up to six weeks in a non-human primate can remain patent and begins to integrate with the host tissue. Unfortunately, ongoing evaluation in the primate model have shown that the model seems to be limited due to mismatch of vessel size and other technical considerations so that more than six weeks data are not obtainable at this time. Still, this data provides significant basis for the expectation that as a long-term clinical implant the graft could demonstrate patency as well as significant repopulation and remodeling by the surrounding host tissue. Such a graft in the off the shelf configuration described in this report would be a desirable alternative to current interventions for hemodialysis access; occluding non-remodeling synthetic PTFE grafts and inconsistent native fistulas.

The progress made in this effort provide a substantial basis for the advancement of these processed vascular tissue grafts as candidate grafts for vascular combat casualty care.

References:

N/A

Employees:

LifeCell Employees that have received pay through this research grant:

Nathaniel Bachrach
Elaine Blessing
Larry Boerboom
Marianne Edwards
Mark Hayzlett
Michael Honey
Maryellen Sandor
Mallika Suresh
Hsin-Chien Tai
Hui Xu

Appendices:

Appendix A – Transmission Electron Microscopy of Vein Grafts

Appendix B – Cryoprotection of Undissected Umbilical Vessels

Appendix C – Establishing a Process for DNA removal with rhDNA

Appendix D – Biocompatibility Testing

Appendix E – Quality Plan for Vascular Grafts

Appendix F – Effect of Gamma Irradiation on the Human Umbilical Vein Graft

Appendix G – The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Appendix H – Matrix Integrity of Vascular Grafts after -80°C Freeze Thaw

Appendix I – Development of Preservation Methods for Human Umbilical Vein Grafts

Appendix J – In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

Appendix K – Stability of Glycerol Preserved Human Umbilical Vein Grafts

Appendix L – In Vivo Evaluation of Processed Human Umbilical Vein and Artery in a Small Animal Model (Summary Report)

Appendix M – In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

Appendix N – In Vivo Assessment of Umbilical Artery Graft in a Porcine Model – Feasibility Study (Summary Report)

Appendix O – Evaluation of Umbilical Artery Tissue Dissection

Appendix P – Porcine Umbilical Vein Acute Implantation Study (Summary Report)

Appendix Q – Canine Umbilical Vein Acute Implantation Study (Summary Report)

Appendix R – In Vivo Assessment of Umbilical Vein Grafts in a Porcine Model – Feasibility Study (Summary Report)

Appendix S – Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

Appendix A - Transmission Electron Microscopy of Vein Grafts

Objective

The objective of this electron microscopy study was to evaluate the effects of chemical decellularization and freeze-thaw of the tissue. Processed and unprocessed umbilical vein tissue was compared by electron microscopy to determine that there is a removal or substantial destruction of cellular organelles that could provide the potential for an immunogenic response and retention of the fundamental components and structures of the tissue.

The test article in this evaluation was umbilical vein processed by standard methods (n=5 donors, longitudinal and cross section samples). The control article was umbilical vein received and cryopreserved but with no further processing (n=1 donor, longitudinal and cross section samples).

Results

The intima of the unprocessed veins consists of an endothelial layer, a basement membrane, an internal elastic lamina and collagen (Figure 1a&b) In the processed tissues the endothelial layer had successfully been removed (Figure 1c). Only very occasional cellular debris was reported.

In the control tissue the basement membrane was present and fairly continuous covering about 75% of the lumen surface and an internal elastic lamina was evident (Figure 1b). There was over 50% basement membrane coverage in all (5) veins evaluated. Three of the five veins had 70% + coverage, which is similar to that seen in the fresh samples. In some cases there were small tears suggestive of damage due to mechanical handling either as part of the tissue process or processing the tissue for electron microscopy.

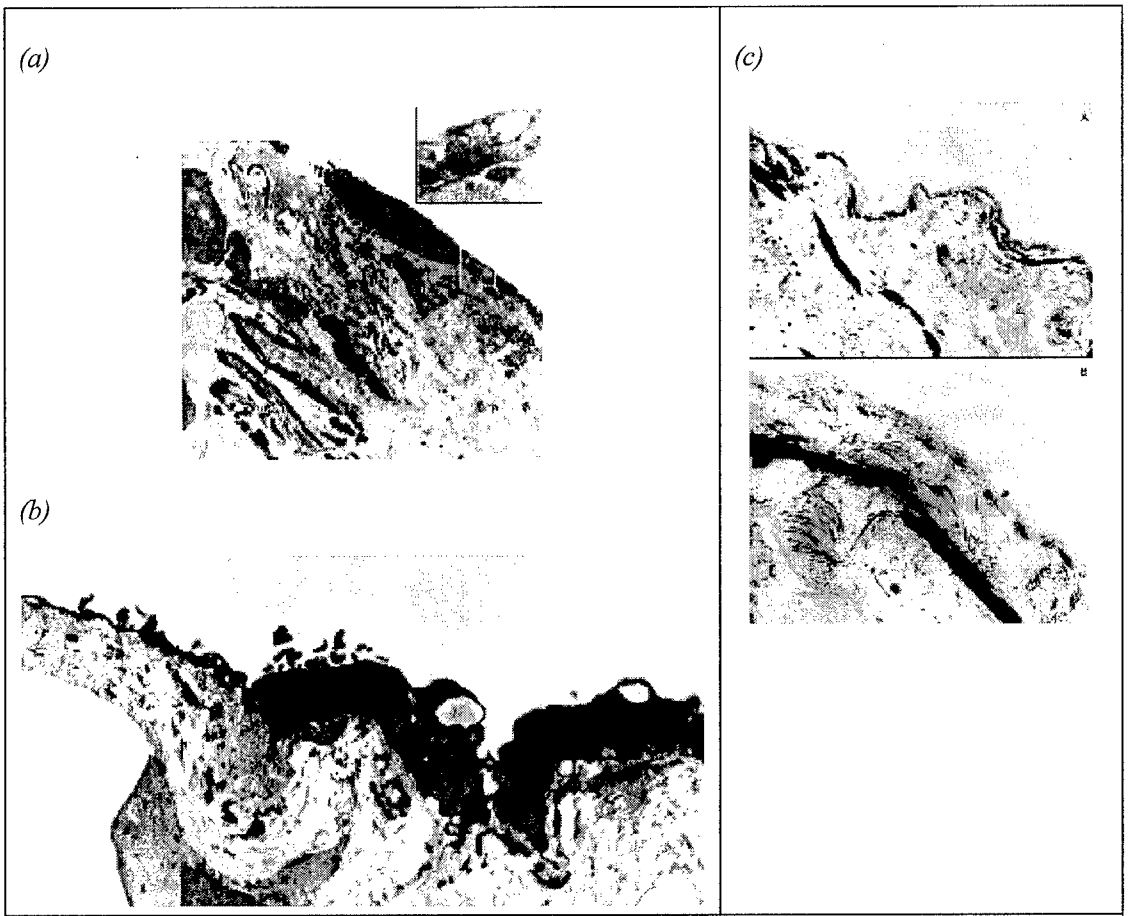


Figure 1: Unprocessed human umbilical vein has (a) intact endothelial cells (3,000x) containing a Weibel-Palade body and (b) intact, a continuous basement membrane, elastin, and collagen, (4,000x). (c) UVG has no endothelial cells but has an intact basement membrane and supporting extracellular matrix (top @ 3,000x, bottom at 10,000x)

The control tissue media was thick, compact, and rich in smooth muscle cells (SMC) that were well preserved SMC with few organelles, a central nucleus, a fine network of filaments, dense bodies, dense plaques, caveolae, and a continuous basal lamina (Figure 2a). In the processed tissue the SMC nuclei and organelles were successfully removed. No convincing cell remnants (recognizable organelles) remained (Figure 2b). The basal lamina and the filamentous network containing smooth muscle filaments, dense bodies, and dense plaques, remained. These remaining cell components have been defined as cellular debris.

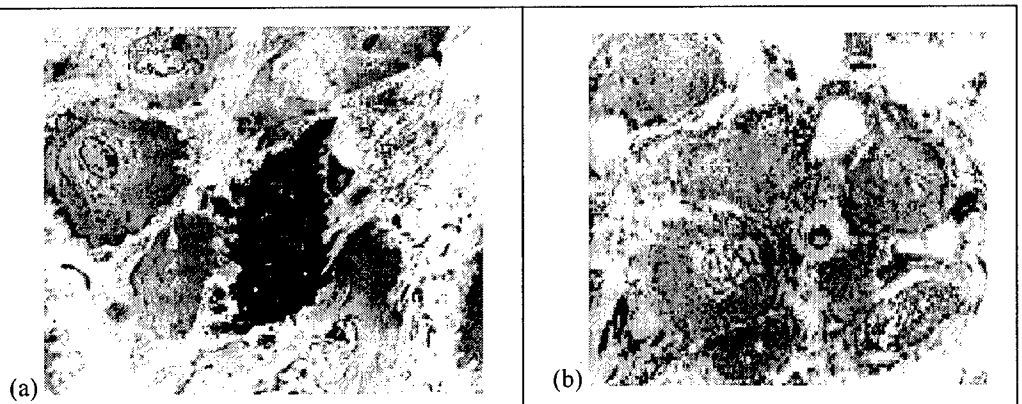


Figure 2: Smooth muscle cells of the unprocessed umbilical vein (a) show centrally located nuclei, filamentous network and cell organelles (2,500x). In the processed tissue (b) the cell organelles are unrecognizable (3,000x).

Also present in the extracellular matrix were occasional wisps of elastin, collagen, fine filaments and ground substance. Collagen periodicity was evident and maintained in processed tissue (Figure 3).

Figure 3: Lifecell processed umbilical vein. Collagen fibers in UVG demonstrating typical periodicity (35,000x).

While there is some controversy in the literature[1] (Bergman, Nanaev) regarding the presence of a adventitial layer in the vessels of the umbilical cord it was noted in our observations as a thin layer consisting primarily of collagen. The few cells seen in the adventia and in the surrounding Wharton's Jelly were substantially removed or reduced to debris.

[1] Bergman, R.A., et al. *Atlas of Microscopic Anantomy: Section 13 – Female Reproductive System, Plate 13.261 Umbilical Cord.* Virtual Hospital, University of Iowa Health Care. 1992 – 2003. and Nanaev, A.K. et al. *Stromal Differentiation and Architecture of the Human Umbilical Cord.* Placenta (1997), 18, 53-64.

Conclusions

The most significant difference in tissue morphology between the fresh umbilical veins and the UVG is the successful removal of the endothelium, while maintaining a relatively intact basement membrane. Additionally, the cellular components of the media, adventitia, and Wharton's Jelly, that may have the potential to produce an immune response, were either removed or disrupted beyond recognition. These cellular components include the nucleus and nuclear components, the cell organelles, and the cell membranes (excluding the basal lamina).

Cell cytoskeletons contribute to mechanical integrity of the matrix in tissues with a high cell to tissue ratio. It is believed that the filamentous network of the cells that remains in the UVG is important in maintaining tissue structure.

Appendix B - Cryoprotection of Undissected Umbilical Vessels

Test articles:

- Freshly isolated vein and artery tissues.
- Vein and artery tissues isolated after the whole (undissected) cord treated with 50% (w/w) X-101 for 4 hours.
- Dissected vein and artery tissues treated with 50% X-101 overnight (~ 19 hours)
- Vein and artery tissues isolated after the whole (undissected) cord treated with 50% (w/w) X-101 overnight (~ 22 hours)
- Freshly isolated vein and artery tissues partially dehydrated for ~16 hours under 91% RH in a closed container.

Note: Vein and artery tissues included some adherent Wharton's jelly.

Differential scanning calorimetry (DSC) analysis was performed using the following temperature program. Each sample was cooled at 1 °C/min from 10°C to –60°C, held for 10 min at –60°C, and then warmed at 2 °C/min from –60°C to 20°C. Actual final temperature was roughly –64°C before warming.

Results:

Melting enthalpy was calculated for the various test samples. The data is presented below.

	Melting Enthalpy	
	Vein (J/g sample)	Artery (J/g sample)
Fresh tissue	257.1	268.4
Whole cord, 4-h treatment	150.1	192.4
Dissected tissue, ~19-h treatment	93.8	77.0
Whole cord, ~22-h treatment	71.2	79.7
Partially dried under 91% RH	186.3	220.5

DSC also was used to determine the onset temperatures for T_h and T_g of samples (onset temperature). The data are presented below:

	T_h (°C)		T_g (°C)	
	Vein	Artery	Vein	Artery
Fresh tissue	-14.7	-13.4	-45.0, -28.1	No sample
Whole cord, 4-h treatment	-16.3	-12.6	-23.3	-27.2
Dissected tissue, ~19-h treatment	-14.9	-18.0	-20.2	-20.7
Whole cord, ~22-h treatment	-18.9	-23.6	-21.2	-22.4
Partially dried under 91% RH	-14.5	-13.1	-36 (?)	-38 (?)

(?) not clearly identifiable on the thermograms

After DSC measurement, water content was determined by gravimetric method. The data are presented below.

	Vein (g/g dw)	Artery (g/g dw)
Fresh tissue	7.4	9.1
Whole cord, 4-h treatment	1.7	2.8
Dissected tissue, ~19-h treatment	0.8	0.7
Whole cord, ~22-h treatment	0.6	0.7
Partially dried under 91% RH	4.0	3.7

Conclusions:

The data demonstrate that the vessels in the umbilical cord are cryoprotected to the same extent as dissected tissue that has been incubated for less time.

Objective:

To assess the activity and effectiveness of Pulmozyme at various concentrations, temperatures, and lengths of incubation.

Methods and Results:

Small segments (approximately 4 x 9 x 1 mm) of freshly harvested sheep carotid artery were decellularized according to the initial steps of the usual processing protocol. Each was then subjected to a treatment with enzyme solution containing either 2, 5, or 10 μg/mL Pulmozyme with all other component concentrations being kept the same, and each of these was incubated for either 4 hours or overnight at either room temperature or 37°C. Following this step, the samples were both assayed using Qiagen DNeasy kit (n=3) and assessed histologically (n=1) for residual nuclear remnants. Test samples were compared to decellularized tissue not subjected to the enzyme step and to control samples treated with bovine DNase at 37°C overnight.

Quantification of remnant DNA indicated that the use of solutions containing 2 μg/mL Pulmozyme were much less effective than those containing 5 or 10 mg/mL, and, as expected, each of the three solution concentrations was significantly less effective when used at room temperature than when incubated at 37°C. Overnight incubation, however, did appear to increase the effectiveness of Pulmozyme when used at room temperature as compared to its use for 4 hours only (Figure 2).

Histological observations supported those findings. It was also evident that 37°C incubations were more effective than room temperature incubations, and overnight incubations more effective than 4 hour incubations (data not shown). Conditions that appeared to remove all nuclear remnants histologically included the control solution containing 150 U/mL bovine DNase I incubated overnight at 37°C, the solution containing 2 μg/mL Pulmozyme incubated at 37°C overnight, solutions with 5 μg/mL Pulmozyme incubated at 37°C for either 4 hours or overnight, and solutions containing 10 μg/mL Pulmozyme incubated at 37°C for either 4 hours or overnight and incubated at room temperature overnight.

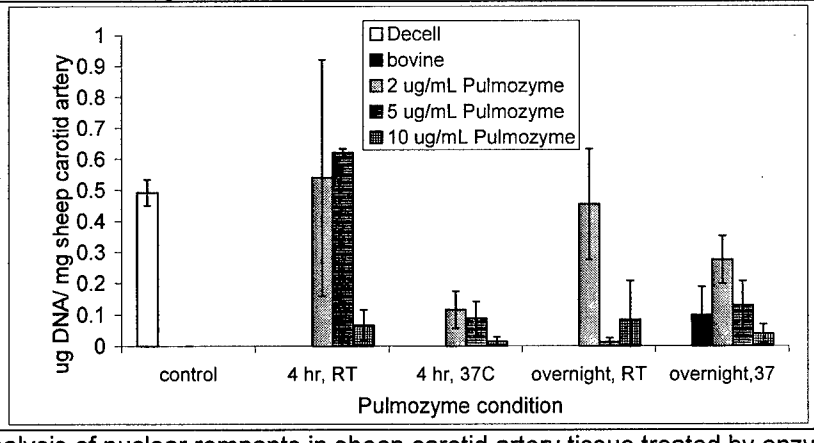


Figure 2: Analysis of nuclear remnants in sheep carotid artery tissue treated by enzymatic digestion under various conditions. Results obtained by DNA assay

To determine the optimal concentration of Pulmozyme to use in processing of both umbilical artery and vein, the safest and most effective treatment from the previous experiment (tissue incubated with10 □g/mL Pulmozyme overnight at room temperature – Figure 3b) was compared to higher concentrations of Pulmozyme, 20 and 30 □g/mL, added to the enzyme solution. Larger tissue segments (tubular segments, 4.5 cm long for umbilical veins and 6 cm long for umbilical arteries) were used for this experiment to get a more accurate assessment of how the enzyme would perform when used with full length grafts. The wet weight of tissue to overall enzyme solution volume ratio was also varied with ratios of 20, 25, and 30 mg tissue per mL of solution for umbilical arteries and 40, 50, and 60 mg tissue per mL solution for umbilical veins. Each of the test conditions was assessed under room temperature conditions overnight and compared to a bovine DNase I control incubated at 37°C. Residual DNA was again assessed both by DNA assay (n=1) as well as histology (n=1).

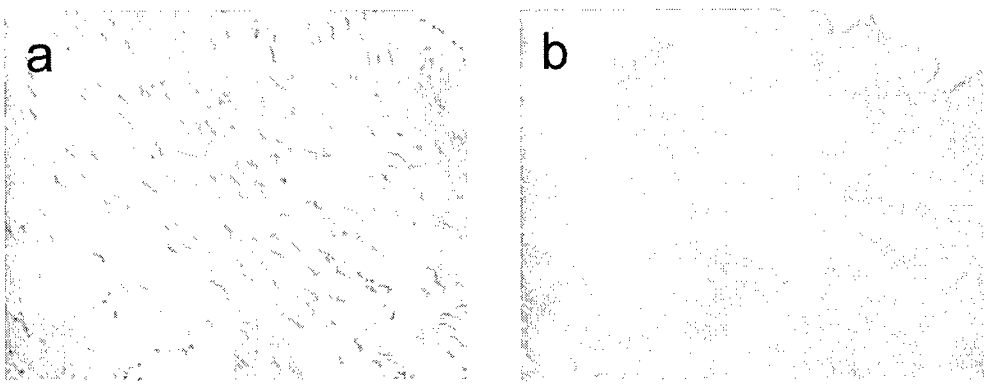


Figure 3: Histological assessment of (a) untreated sheep carotid artery tissue and (b) tissue treated with enzyme solution containing 10 μg/mL Pulmozyme at room temperature overnight.

When evaluated via DNA assay, all conditions tested appeared to decrease DNA content relative to unprocessed control for both umbilical artery and umbilical vein (Figure 4a, 5a) and results were comparable to those obtained with bovine DNase I at 37°C. Neither concentration of Pulmozyme nor tissue weight to solution volume ratio, however, appeared to show any trend.

Histologically, arteries appeared to be somewhat decellularized with 10 μg/mL Pulmozyme, with less nuclear remnants visible with increasing concentrations (20 and 30 μg/mL). Although no nuclei could be clearly identified in the artery segments treated with 30 μg/mL Pulmozyme, a faint blue cast of hematoxylin staining could be seen within the tissue, indicating the presence of scant amounts of residual DNA (Figure 4b). Umbilical veins showed similar results to umbilical arteries histologically, but without a blue cast to the tissue with the use of 30 μg/mL Pulmozyme (Figure 5b).

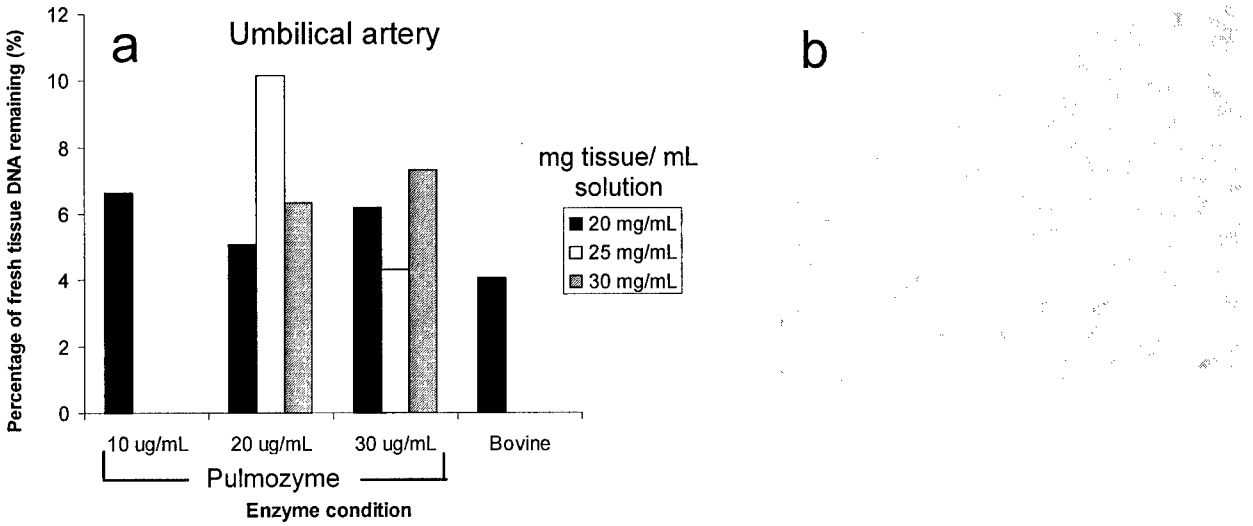


Figure 4: Assessment of umbilical artery nuclear remnants following nuclease digestion treatment. (a) DNA assay, (b) histological section.

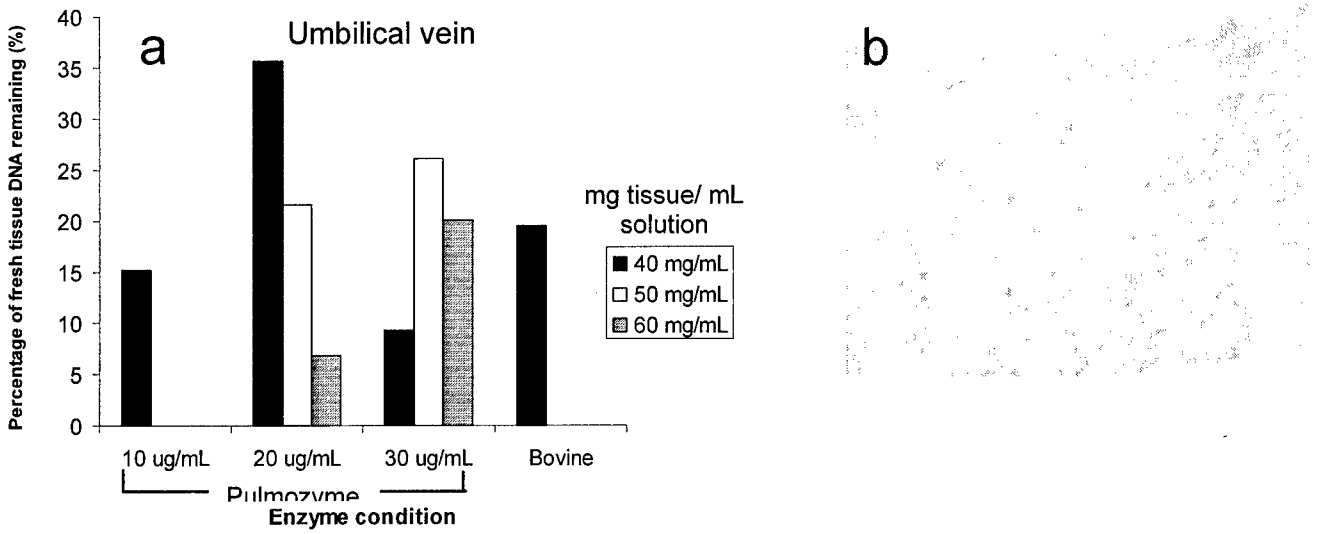


Figure 5: Assessment of umbilical vein nuclear remnants following nuclease digestion treatment. (a) DNA assay, (b) histological section.

Conclusion:

To ensure that all possible nuclear material would be removed from processed tissues, the concentration of Pulmozyme to be used with both umbilical arteries and veins was increased to 40 µg/mL enzyme solution and the tissue weight to solution volume ratio was kept at a minimum – 20 mg tissue/mL enzyme solution for umbilical arteries and 40 mg/mL for umbilical veins. Vessels treated with these conditions were consistently found to be decellularized histologically.

Pyrogenicity:

Test System

A previously established test system designed to detect the presence of chemical pyrogens in solid materials was employed. In this model a test article is extracted in saline and the resulting solution is injected intravenously in New Zealand White Rabbits. The Rabbits are monitored for 3 hours for any febrile response to the injection. A febrile response is defined as an increase in temperature = 0.5°C.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours' detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of graft leachables to elicit a pyrogenic response was evaluated.

Each tissue sample was extracted @ 37°C for 24 hr in 4g tissue/80ml 0.9% saline and then 10ml extract per kg body weight of the animal was injected into each of three rabbits rabbit.

Results

The data are summarized in Table 1 and demonstrate that the UVG is not pyrogenic.

Table 1: Summary data of pyrogenicity testing.

DNase Soak Time (hours)	UVG Lot #	Maximum Remperature Rise (°C)		
		Animal		
		1	2	3
18	SFH-4	0.0	0.0	0.1
18	SFH-15	0.0	0.0	0.0
18	SFH-20	0.0	0.0	0.4
24	SFH-3	0.2	0.0	0.0
24	SFH-6	0.0	0.0	0.0
24	SFH-12	0.0	0.0	0.3

Cytotoxicity:

Test System

A previously established test system designed to determine the cytotoxicity of solids and extracts was employed. The test consists of a confluent monolayer culture of L929 mouse fibroblasts. The culture is maintained in the presence of the test article for an additional 48 hours at 37°C and evaluated under a microscope biological reactivity using cytochemical stains. A sample is considered cytotoxic if it incites more than a mild response.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of the cytotoxicity of the graft and its leachables was evaluated.

Two different test articles were used; MEM elution for leachables, and agar diffusion to assess "direct contact". The MEM elution test samples were derived from extracting 1cm2 section of tissue in MEM @ 37°C for 24 hr in 10ml. Agar diffusion test samples were $1cm^2$ sections of tissue placed directly against the 0.5-2g/ml agar layer covering the monolayer culture. Each test was performed in triplicate.

Results

The data are summarized in Table 2 and demonstrate that the UVG is not cytotoxic as reactivity ratings were zero for all samples.

Table 2: Summary data of cytotoxicity testing.

DNase Soak Time (hours)	UVG Lot #	Agar diffusion			MEM elution		
		Plate			Plate		
		1	2	3	1	2	3
18	SFH-4	0	0	0	0	0	0
18	SFH-15	0	0	0	0	0	0
18	SFH-20	0	0	0	0	0	0
24	SFH-3	0	0	0	0	0	0
24	SFH-6	0	0	0	0	0	0
24	SFH-12	0	0	0	0	0	0

Hemolysis:

Test System

A previously established test system designed to determine the hemolytic potential of solids and extracts was employed. The test consists of a concentrate of human red blood cells in 0.9% saline. The concentrate is incubated in the presence of the test article for a 1 hour at 37°C. After incubation the optical density of the concentrate is evaluated using a spectrophotometer to assess the degree of cell lyses.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of the cytotoxicity of the graft and its leachables was evaluated.

Two different test articles were used; extracted samples for leachables, and intact tissue ta assess direct contact. The extraction test samples were derived from extracting 0.1g of tissue @ 37°C for 24 hr in 1.5ml of 0.9% saline. Direct contact test samples were 0.1g of tissue immersed directly in the red blood cell concentrate. Each test was performed in triplicate. Maximum acceptable levels of hemolysis were established as 5%.

Results

The data are summarized in Table 3 and demonstrate that the frozen UVG is not hemolytic as the percent hemolysis did not even approach the 5% level.

Table 3: Summary data of cytotoxicity testing.

DNase Soak Time (hours)	UVG Lot #	Percent Hemolysis (%)					
		Direct contact			Extraction		
		Tube			Tube		
		1	2	3	1	2	3
18	SFH-4	0.39	0.08	0.70	0.00	0.00	0.47
18	SFH-15	0.08	0.78	0.16	0.00	0.08	0.00
18	SFH-20	0.00	0.00	0.00	0.00	0.00	0.47
24	SFH-3	0.39	0.31	0.08	0.08	0.00	0.00
24	SFH-6	0.00	0.00	0.00	0.00	0.00	0.00
24	SFH-12	0.00	0.00	0.00	0.00	0.00	0.00

Appendix D - Biocompatibility Testing

Donor Screening

As specified by AATB all donors are assessed for risk of infectious diseases using a Donor Medical History & Behavioral Risk Assessment Questionnaire and a physical evaluation. The following serological testing is performed: HIV I&II, Hepatitis B&C, HTLV I&II, and Syphilis, all of which must be negative or the tissue is rejected for processing. A pathologist does a direct visual review of all umbilical cords before they are shipped to LifeCell. Tissue is released and approved for processing by LifeCell's medical director following review of all donor information.

Microbiological testing

All umbilical cords have 0.5cm samples cut before immersion in antibiotic solutions and sent for microbiological testing with enumeration. Tissue that is found to be positive for spore forming microbials is excluded from processing.

All lots have a 0.5cm sample taken that will be destructively tested in its entirety for sterility (14 day) in at least 300ml of solution as has been determined to be required for normal growth in Bacteriostatic-Fungistatic testing. Any positive growth results in graft rejection.

Processing

In order to significantly reduce the tissue bio-burden and minimize chance of infection, the tissue is exposed to various antibiotics covering a broad spectrum of activity and an antifungal agent. Following procurement, the tissue is shipped and stored in a solution containing antibiotics. The tissue is not in this solution for more than 30 hours. During processing at Lifecell the tissue is exposed to solutions that contain antibiotics and an antifungal agent.

Contamination and Cross contamination

All measures in place for the prevention of contamination and cross contamination are delineated in a standard operating procedure. The procedure requires that tissues from different donors may not be combined during processing. It prescribes that reusable instruments, equipment and supplies must be disinfected, cleaned, and sterilized between uses. Also, tissue is only exposed in controlled Class 100 areas and only approved disinfectants may be used as cleaning agents for those areas. Finally, the procedure directs additional standard procedures for biohazard waste disposal and management should that be needed.

A class 100 clean room is used to perform the tissue dissection. The qualification of this processing environment has been completed and is maintained in a controlled state using standardized procedures for operation, environmental monitoring and routine cleaning, hygiene and gowning. The area is annually certified.

All other processes involving tissue exposure are carried out in LifeCell's main cleanroom area and subject to the controls currently in place that define cleanroom hygiene, gowning, and cleaning and cleanroom environmental monitoring for those production areas.

Quality control testing

Grafts that are produces need to have some testing provided to provide an assurance that the vessels have been processed appropriately. The tests and pass/fail criteria chosen to be used for quality testing on every lot of vascular graft were developed and are described below.

The process calls for the removal of MHC I&II in the detergent incubation. The effectiveness will be tested using immunohistochemistry (IHC) to look for MHC in cryosections. As these are antigenic components of the cells, these tests also serve a safety function. Although the amount of MHC removal that is critical is not known, the experience with the process to date has been that removal is achieved. Therefore, a process drift will be noted by positive presence of MHC, and consequently a positive MHC stain will not be acceptable.

The process also calls for the substantial removal of the nuclear material often left after the detergent step, using a DNase solution. The presence of nuclear material has been evaluated using hematoxylin & eosin staining. Removal of DNA is not functionally critical as non-decellularized allografts are used clinically however, the experience with the process to date has been that removal is substantially achieved and its removal is believed to be beneficial. Therefore, a process drift will be noted by presence of significant cell associated hematoxylin staining and will constitute a failure.

Qualitative assessment of the matrix and basement membrane will also be performed by the project leader as an extra check that there has not been extreme damage to either from unexpected causes.

I. Objective(s)

1.1. To determine the effect of different γ-irradiation schemes on the following: processing (ie difficulty of dissection, pliability, etc), X-101 penetration, histology, electron microscopy, suture retention, circumferential tensile compliance, and test to failure.

II. Protocol

1.2. Seven umbilical veins were each divided into the following conditions:

W: Control, no γ-irradiation
X: pre-dose of 12 kGy γ-irradiation
Y: final dose of 12 kGy γ-irradiation
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation

1.3. Samples were packaged for γ-irradiation as per the protocol and stored at approximately -80°C until the time of testing.

1.4. Five out of the seven sets of grafts were processed for testing as described in section 4.5 below. The remaining two were retained

1.5. Prior to testing, samples were removed from storage, the grafts were removed from the packaging and placed into 0.9% NaCl (10ml/cm of graft) After 30 minutes' agitation on a shaking platform (ambient temperature), the saline was drained and replaced, and the grafts placed back on the shaking platform for an additional 30 minutes.

1.6. After the second saline wash, sections of grafts were submitted for testing as per the protocol.

1.7. Due to insufficient sample length for graft 12909, only one set of duplicate measurements could be performed for the X-101 infiltration test, rather than the protocol-specified two measurements on two subsample pieces.

III. Results

1.8. Processing Evaluation

1.8.1. Pre-dose γ–irradiation (treatment X) samples took an additional 30 minutes to thaw in the water bath. The arteries were easier to dissect and did not tear or shred.

1.8.2. Pre-dose γ–irradiation (treatments X and Z) resulted in darker colored grafts compared to no irradiation (W) and post-dose irradiation (Y).

1.8.3. Post-dose γ–irradiation (treatments Y and Z) resulted in collapsed and flattened grafts in which the Whartons Jelly (WJ) had shrunk and was indistinguishable from vein tissue. Although the WJ rehydrated in the usual way, the lumen collapsed during the subsequent handling.

1.8.4. Treatment W samples were found to be slimier than usual and had a foul odor upon rehydration.

1.9. Mechanical Testing

1.9.1. The data from the mechanical tests are summarized in Table 1 below.

1.9.2. The data all fall within the same range regardless of γ-irradiation treatment.

Table 1: Mechanical Testing Data (n=5; avg ± std dev)

Treatment	Suture Retention Strength (N)	Compliance (N/%strain)[1]	Break (N)
W: Control, no γ-irradiation	4.39 ± 1.91	6.08 ± 1.58	18.95 ± 7.49
X: pre-dose of 12 kGy γ-irradiation	4.28 ± 0.80	9.21 ± 5.08	15.83 ± 5.66
Y: final dose of 12 kGy γ-irradiation	4.95 ± 3.06	7.56 ± 3.48	16.66 ± 6.76
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation	4.25 ± 0.90	8.94 ± 8.99	16.05 ± 4.07

1.10. X-101 Infiltration

1.10.1. Two random samples from each of the four treatment groups were tested for X-101 infiltration using a thermogravimetric measurement.

1.10.2. No statistical comparisons were made due to the low sample number (n=2) for each treatment. The data, summarized in Table 2 below, show no differences between the four groups.

Table 2: X-101 Infiltration Data

	Graft ID	%X-101 *
W	12575A	31
	12909A	32
X	12575B	29
	12909B	31
Y	12575A	32
	12909A	32
Z	12575B	30
	12909B	31

* The data represent an average of two measurements for each graft.

1.11. Histology (see section 9.1)

1.11.1. Gamma irradiation did not affect either the internal elastic lamina or basement membrane.

[1] For the compliance test, three samples were tested from each graft. The average of the three measurements for each of the five grafts was then used to generate the data reported in Table 1.

1.11.2. Condensed collagen in the matrix was present in all samples that had been γ-irradiated. However, it was more prevalent in the pre-dissection samples (treatment X).

1.11.3. Post-dissection irradiation (treatment Y) appears to produce more holes in the Wharton's Jelly compared to control (W). However, holes were not observed in those that were double-dosed (Z).

1.11.4. The single-dosed γ-irradiated samples (treatments X and Y) showed more separation compared to the control (W). However, the double-dosed samples (Z) did not appear worse than either X or Y.

1.12. Electron Microscopy (ref. 3.6. above)

1.12.1. The electron microscopy analysis revealed "subtle" differences between the four treatment groups. However, no definitive conclusions could be drawn as to which of the γ-irradiation schemes may be deleterious to the tissue.

1.12.2. Samples in treatment W (the control group) showed a higher level of enthothelial cell debris and cell debris in the empty spaces compared to historical samples. The electron microscopist speculated that the decellularization step may not have been as complete as expected.

1.12.3. Treatment in the pre-dissection samples (X) appears to be better compared to either Y or Z based on basement membrane coverage and spacing/separation of tissue.

1.13. Rehydration

1.13.1. Two samples from each of two lots, 12575A and 12909A, were tested.

1.13.2. Table 3 contains some excerpted data from the report on those tests.

Table 3: Rehydration Data

Treatments	Packaging method before rehydration analysis	Hydration after γ treatment (g water per g dry tissue) *	Hydration after full rehydration (g water per g dry tissue)
W	Tyvek/Foil bag	15.6 ± 1.9	25.0 ± 1. 9
X	Tyvek/Foil bag	12.6 ± 0.9	16.7 ± 1.2
Y	Tyvek/Cassette	19.0 ± 1.6	16.7 ± 0.7
Z	Tyvek/Cassette	13.3 ± 0.3	14.5 ± 1.1

* Data represent the mean ± standard deviation of four measurements (two samples from each of two lots)

1.13.3. The data show that both pre- and post-dose γ-irradiation resulted in an approximately 35% decrease in rehydration compared to the control which was not irradiated.

1.13.4. There was an additional 13% decrease in hydration for the double-dosed grafts (Z) compared to the single-dosed grafts (X and Y).

IV. Analysis

A 2-way ANOVA test with replication was performed on the mechanical testing data at $p<0.05$. The factors were the pre- and post-dose γ-irradiation. The results are shown in Table 4 for compliance, Table 5 for break, and Table 6 for suture strength. The data show no significant difference among the treatments.

Table 4: 2-Way ANOVA Results for Compliance (N/% strain)

Effect	Repeated Measures Analysis of Variance compliance Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	1263.89	1	1263.89	35.9195	0.00390
Error	140.75	4	35.19		
PRE-DOSE	25.43	1	25.43	1.0676	0.35987
Error	95.26	4	23.82		
POST-DOS	1.82	1	1.82	0.0486	0.83625
Error	149.51	4	37.38		
PRE-DOSE*POST-DOS	3.85	1	3.85	0.1541	0.71464
Error	99.80	4	24.95		

Table 5: 2-Way ANOVA Results for Break (N)

Effect	Repeated Measures Analysis of Variance break (N) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	5693.63	1	5693.63	65.3947	0.00127
Error	348.26	4	87.07		
PRE-DOSE	17.50	1	17.50	0.6759	0.45717
Error	103.58	4	25.90		
POST-DOS	5.38	1	5.38	0.4876	0.52347
Error	44.11	4	11.03		
PRE-DOSE*POST-DOS	7.88	1	7.88	0.2986	0.61383
Error	105.51	4	26.38		

Table 6: 2-Way ANOVA Results for Suture Strength (N)

Effect	Repeated Measures Analysis of Variance suture strength (N) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	399.08	1	399.08	40.13	0.00
Error	39.78	4	9.95		
PRE-DOSE	0.84	1	0.84	0.26	0.63
Error	12.78	4	3.19		
POST-DOS	0.35	1	0.35	0.40	0.56
Error	3.52	4	0.88		
PRE-DOSE*POST-DOS	0.44	1	0.44	1.05	0.36
Error	1.67	4	0.42		

V. Conclusions

1.14. The pre-dose γ-irradiation appeared to affect the ease of arterial dissection. However, it is not known at this time whether the extended thawing time or the darker colored graft will have a significant effect on functional performance.

1.15. The *in vitro* characterization data from both the mechanical testing and the microscopic analysis show that no significant differences between any of the treatments.

1.16. The reduced rehydration observed in the post-dose (Y) and double-dosed (Z) conditions appears to verify the visual observation that the Wharton's Jelly appeared shrunken and collapsed.

1.17. This study has yielded some clues about the effect of γ-irradiation on the umbilical vein graft. However, it is difficult to judge at this time which treatment scheme can be considered optimal. Therefore, follow-up studies in which higher γ-irradiation dosages are utilized are recommended.

VI. Appendices

1.18. Histology Data

Table 7: Histological Evaluation Results

		H & E						
Slide #	**Cord #**	**Group**	**Matrix**	**Holes**	**Separation**	**PAS**	**Verhoeffs**	**Comments**
5392-1	12575	W	A	C	A	B	B	
5392-2	12575	X	C	C	C	B	B	
5392-3	12575	Y	D	D	C	B	B	
5392-4	12575	Z	D	A	C	B	B	
5392-5	12909	W	B	A	B	B	B	
5392-6	12909	X	C	A	D	B	A	
5392-7	12909	Y	A	D	C	B	A	
5392-8	12909	Z	D	B	A	B	B	
5392-9	12799	W	D	B	B	B	B	
5392-10	12799	X	C	A	C	C	B	
5392-11	12799	Y	D	A	C	C	B	
5392-12	12799	Z	C	A	B	B	B	
5392-13	12798	W	A	C	C	B	A	Fell between C & D separation
5392-14	12798	X	C	A	C	B	B	Fell between C & D separation
5392-15	12798	Y	C	A	D	A	A	Fell between C & D separation
5392-16	12798	Z	B	A	D	B	B	Fell between C & D separation, "Blown Apart"
5392-17	12820	W	C	A	A	B	B	
5392-18	12820	X	C	A	C	B	B	Fell between C & D separation
5392-19	12820	Y	C	B	D	B	B	Fell between C & D separation, "Blown Apart"
5392-20	12820	Z	D	A	C	B	B	

Group Descriptions	
W	Control
X	12 kGy Pre-Dose
Y	12 kGy Post-Dose
Z	12 kGy Pre and 12kGy Post Dose

Table 8: Histology Rankings Definition

Description of categories		
Matrix	A	Nothing
	B	Damage (Broken or Torn)
	C	Areas of Condensed Matrix
	D	Both Damaged and Condensed areas
Holes	A	Nothing
	B	In media
	C	Very large in WJ
	D	In both Media and WJ
Separation	A	Typical Control Levels
	B	A little worse, more separation
	C	More throughout the media and into WJ
	D	Everywhere
PAS	A	Complete
	B	In-complete (at least one break)
	C	More In-complete (torn even on H&E)
Verhoeffs	A	Complete
	B	In-complete (split or gaps)

Appendix G - The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

I. Objective(s)

1.1. To determine the dose response of a cryoprotected[1] and frozen graft to γ-irradiation (0, 12, 18, and 24 kGy).

1.2. To determine the effect of ambient temperature storage (30 days) on non-cryoprotected grafts that had been γ-irradiated at 12 kGy.

1.3. To determine the equivalence of ambient temperature stored, non-cryoprotected grafts to cryoprotected grafts stored at -80°C (eg storage solution).

1.4. To evaluate the effect of accelerated storage on cryoprotected grafts stored under ambient conditions.

II. Protocol(s)

1.5. Eleven umbilical cords were processed. The grafts used and associated test conditions are summarized in Table 1, Table 2, and Table 3 below.

Table 1: Cryoprotected Grafts Stored 30 Days

			Time 0, -80°C		D30, ambient	
Lot	Graft ID	Storage Sol'n	A	B	C	D
1	12698	X-101	0	12	0	12
2	12709	X-101	0	18	0	18
3	12936	X-101	0	24	0	24

Table 2: Non-Cryoprotected Grafts Stored at Ambient Temperature

			Time 0		D30	
Lot	Graft ID	Storage Sol'n	A	B	C	D
4	12976	PBS	0	12	0	12
5	12937	PBS	0	12	0	12
6	13380	PBS	0	12	0	12

[1] In the context of this study, "cryoprotected" refers to grafts that have been equilibrated with X-101 (also referred to as PD-30) and stored at -80°C.

Table 3: γ-Irradiation Dosages (kGy), -80°C Storage Samples (Time 0)

			-80°C Storage			
Lot	Graft ID	Storage Sol'n	A	B	C	D
7	13057	PBS	0	12	12	12
8	12406	X-101	0	12	18	24
9	12499	X-101	0	12	18	24
10	12500	X-101	0	12	18	24
11	12708	X-101	0	12	18	24

1.6. Prior to testing, samples were removed from storage, the grafts removed from their packaging and placed into 10ml saline/cm graft. After 30 minutes' agitation on a shaking platform (ambient temperature), the saline was drained and replaced, and the grafts placed back on the shaking platform for an additional 30 minutes.

1.7. After the second saline wash, sections of grafts were submitted for testing as per protocol requirements.

1.8. Rehydration assay: 1 cm samples from the circumferential test were incubated 24 hrs in saline. Each sample was blotted dry and weighed to obtain the initial wet weight. The samples were then vacuum dried at 107°C for 30 hrs, after which the dry tissue weight was obtained. The rehydration was determined as the ratio of g water to g dry weight.

III. Results

1.9. Processing Evaluation (notebook 224-23)

1.9.1. White dots were observed in lots 2, 3, 8, 9, 10, and 11 immediately prior to rehydration and disappeared thereafter. In the cases of lots 8-11, the dots were more prevalent with increasing γ-irradiation dose.

1.9.2. Although no color change was evident immediately after γ-irradiation , after 30 days' storage under ambient conditions, γ-irradiated cryoprotected grafts became darker in color compared to equivalently stored controls. At least for these samples, the darkness of the color change did not appear to be correlated with γ-irradiation dosage.

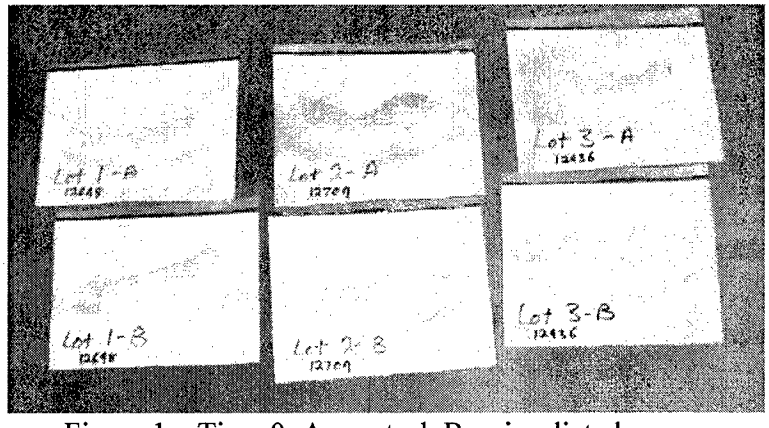


Figure 1a: Time 0; A=control, B=γ-irradiated

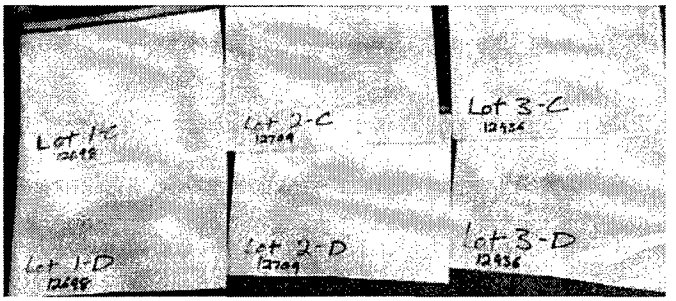


Figure 1b: 30 days; C=control, D=γ-irradiated

Figure 1: Color Change with 30 Days' Storage of Cryprotected Grafts Under Ambient Conditions γ-irradiation dosages: lot 1 = 12 kGy, lot 2 = 18 kGy, and lot 3 = 24 kGy

1.9.3. γ-irradiated grafts were more difficult to cut for histology and electron microscopy sampling.

1.10. Mechanical Testing

The following statistical tests were performed on the mechanical testing data: A 1-way ANOVA was performed to examine the dose response of cryoprotected grafts to γ-irradiation. A 2-way ANOVA was performed to evaluate the effect of ambient storage on non-cryoprotected grafts. An unpaired t-test was used to compare non-cryoprotected (i.e. PBS at ambient conditions) to cryoprotected grafts (-80°C) (e.g. storage solution). A paired t-test was used to evaluate the effect of accelerated storage (ie ambient conditions) on cryoprotected grafts. Statistical significance was set at $p<0.05$ for all analyses.

1.10.1. Suture Strength (N)

1.10.1.1. Suture strength was tested on 2 cm segments from each graft as per RP-0024. The data are summarized in Table 4 for the ambient storage samples and Table 5 for the -80°C samples.

1.10.1.2. Statistical analyses showed no significant differences in suture strength for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was, however, a significant 2-way interaction between both time and γ-irradiation for the ambient stored non-cryoprotected grafts. That is, the 12 kGy dose did not produce a reduction in suture strength seen with the non-irradiated tissue (see Table 4).

Table 4: Suture Strength (N) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	5.34 ± 1.70 (7)	4.00 ± 1.64 (5)	4.25 ± 1.81 (5)	5.03 ± 1.85 (5)
	30	ambient	3.51 ± 1.43 (3)	3.43 (1)	3.50 (1)	5.02 (1)
PBS	0	ambient	5.84 ± 0.83 (3)	3.99 ± 0.99 (3)		
	30	ambient	1.98 ± 1.16 (3)	4.80 ± 1.93 (3)		

Table 5: Suture Strength (N) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	4.31 (1)	3.77 ± 1.24 (3)		
X-101	5.34 ± 1.70 (7)	4.00 ± 1.64 (5)	4.25 ± 1.81 (5)	5.03 ± 1.85 (5)

1.10.2. Compliance (N/%strain)

1.10.2.1. Three 1 cm segments for each graft were subjected to circumferential testing to assess compliance. The data are summarized in Table 6 for the ambient storage samples and in Table 7 for the -80°C samples.

1.10.2.2. Statistical analyses showed no significant differences in compliance for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was a significant reduction in compliance from γ-irradiation for the ambient stored non-cryoprotected grafts (see Table 6).

Table 6: Compliance Data (N/%strain) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	9.87 ± 2.92 (7)	9.19 ± 4.76 (5)	12.25 ± 4.32 (5)	4.80 ± 1.76 (5)
	30	ambient	7.35 ± 4.56 (3)	10.90 (1)	9.28 (1)	4.97 (1)
PBS	0	ambient	9.67 ± 2.14 (3)	4.18 ± 1.25 (3)		
	30	ambient	12.14 ± 3.45 (3)	5.59 ± 1.40 (3)		

Table 7: Compliance Data (N/%strain) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	14.70 (1)	10.28 ± 4.16 (3)		
X-101	9.87 ± 2.92 (7)	9.19 ± 4.76 (5)	12.25 ± 4.32 (5)	4.80 ± 1.76 (5)

1.10.3. Load at Break

1.10.3.1. The load at break data was collected as part of the circumferential test. The data are summarized in Table 8 for the ambient storage samples and in Table 9 for the -80°C samples.

1.10.3.2. Statistical analyses showed no significant differences in load at break for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was a significant reduction in load at break from storage time for the ambient stored non-cryoprotected grafts (see Table 8).

Table 8: Load at Break Data (N) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	22.93 ± 8.92 (7)	16.97 ± 8.54 (5)	18.98 ± 8.36 (5)	16.61 ± 4.87 (5)
	30	ambient	16.16 ± 7.24 (3)	10.90 (1)	19.54 (1)	19.48 (1)
PBS	0	ambient	21.26 ± 1.81 (3)	18.75 ± 1.60 (3)		
	30	ambient	14.84 ± 1.76 (3)	15.62 ± 3.75 (3)		

Table 9: Load at Break Data (N) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	18.57 (1)	14.18 ± 3.05 (3)		
X-101	22.93 ± 8.92 (7)	16.97 ± 8.54 (5)	18.98 ± 8.36 (5)	16.61 ± 4.87 (5)

1.11. Histology (H&E Staining)

1.11.1. With few exceptions, the differences between treatments appear to be subtle. Therefore, no definitive conclusions can be made at this time. The following is a summary of the findings.

1.11.2. Samples, both non-irradiated and γ-irradiated at 12 kGy, that had been frozen in PBS at -80°C exhibited the most damage to the collagen fibers and holes in the Wharton's Jelly.

1.11.3. Thirty days' storage under ambient conditions for both irradiated and non-irradiated samples did not appear to produce additional damage compared to time 0.

1.12. Electron Microscopy

1.12.1. Data were collected for information only and will be reported separately.

1.13. Rehydration (notebook 229:51-54 and 61)

1.13.1. The rehydration data for the 30 days' ambient storage samples are summarized in Table 10 while those for the -80°C time 0 samples are in Table 11.

Table 10: Rehydration Data (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	29.8 ± 4.2 (7) *	19.0 (1)	14.1 (1)	17.4 (1)
	30	ambient	35.5 ± 2.9 (3)	16.2 (1)	14.4 (1)	15.8 (1)
PBS	0	ambient	25.3 ± 0.9 (3)	13.0 ± 1.8 (3)		
	30	ambient	29.5 ± 2.1 (3)	15.2 ± 1.0 (3)		

* The sample number, n, represents the number of lots tested. Three replicate pieces were tested within each lot.

Table 11: Rehydration Data (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	24.2 (1)	12.3 ± 0.6 (3)		
X-101	29.8 ± 4.2 (7)	16.4 ± 3.7 (5)	16.3 ± 3.4 (5)	15.8 ± 2.4 (5)

1.13.2. The combined data in Table 10 and Table 11 show that, for γ-irradiated samples, neither γ-irradiation dosage nor storage time affects rehydration. However, γ-irradiated samples, whether cryoprotected or stored in PBS, do exhibit reduced rehydration compared to non-irradiated samples. Furthermore, the reduced rehydration is similar regardless of the storage solution type.

1.13.3. The data in Table 10 also show an increased hydration for non-irradiated samples that had been stored for 30 days at ambient conditions in either X-101 or PBS. There may be some subtle changes in the matrix structure for the ambient condition stored samples that allowed for the additional hydration.

IV. Conclusions

The results of the spectrum study showed the following:

1. Gamma irradiation appeared to have the most effect on tissue handling characteristics, especially for the pre-dose samples. Furthermore, although γ-irradiated and ambient stored cryoprotected grafts became yellower and darker in color after 30 days, the color change did not appear to have an effect on the mechanical testing results.

2. Histological analysis showed that storage at -80°C in PBS for both γ-irradiated and non-irradiated samples produced more damage to the collagen fibers and created holes in the Wharton's Jelly. It should be noted that there were subtle differences between all samples examined but no conclusive determinations can be drawn at this time.
3. γ-irradiation dosage does not affect the mechanical properties of cryoprotected grafts at time 0.
4. The use of PBS (ambient storage) did not produce significant changes in mechanical properties compared to cryoprotected grafts at time 0.
5. Ambient storage conditions for cryoprotected grafts did not appear to affect the mechanical properties.
6. Both storage time and γ-irradiation (12 kGy) affected the mechanical properties of non-cryoprotected samples – ie γ-irradiation reduced compliance by 55%, storage time reduced load on break by 24%, and a 2-way interaction in which suture strength dropped by 66% with storage time for non-irradiated samples.

Appendix H – Matrix Integrity of Vascular Grafts after -80°C Freeze Thaw

Three mechanical tests were performed in the evaluation of the frozen grafts as they relate to potential failure modes for the vein for use a hemodialysis graft. In each case the vessels were extracted and processed according to the current methods previously described (Annual report 2002) with exception of final freezing of the tissue at -80°C instead of Freeze-Drying. The tests described here were completed after thawing and rinsing the tissue.

Burst pressure testing

The burst pressure of umbilical veins after processing was evaluated by increasing intraluminal pressure by adding fluid to a vessel with a sealed end. The burst pressure was defined as the maximum pressure the vessel can sustain before a precipitous drop in pressure is measured. Initial burst testing on fresh umbilical vein were performed using a syringe to increase the pressure in the vein manually while pressure was monitored on an in line dial gage. The vessel remained hydrated during the procedure. Subsequently a testing apparatus was designed to provide a means for controlled inflation using a syringe pump and electronic data collection to more accurately record peak pressures. In the latter test the vessel was submerged in a water bath of phosphate buffered saline during the testing. The modified testing method provides for better accuracy and control. However, the protocol is sufficiently different that statistical comparison between the two groups is not appropriate.

Results

The vessel is capable of withstanding several times the normal physiologic peak systolic pressure of 120mmHg. Burst was not recorded on average until 856mmHg with a standard deviation of 288mmHg. The burst pressures ranged from 414mmHg to 1396mmHg (Attachment A-1.1). Results are shown in Table 1. Data from fresh tissue tested at a different time are included in the data table for comparison.

Table 1: Burst Strength of frozen thawed UVG and fresh tissue.

Sample type	Burst Pressure (mmHg)	
	Mean ± SD	Range
Fresh Umbilical Vein (n =8)	1344 ± 600	775 - 2430
Processed Frozen Vein (n=10)	856 ± 288	414 - 1396

SD = standard deviation

Failure Analysis

Graft consistency and the potential for acute aneurysm formation or rupture of the HUV graft when stored as frozen graft and implanted as an access graft is addressed using statistical methods below.

Graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The burst pressure is the best available measure of the likelihood of aneurysm formation due to arterial blood pressures sustained in the vein in vivo. The data are provided in Table 2.

Table 2: Range and confidence intervals for burst pressure

Lot #	Burst Pressure (mmHg)
10746	983
10890	621
10896	1086
10911	414
10917	776
10919	905
11029	1396
11353	931
11355	931
11376	517

Statistic	Resutls (mmHg)
[1]N	10
Mean	856
Lower Confidence limit -95%	650
Lower Confidence limit -99.5%	520
Min	414
Max	1396
Standard Deviation	288

AV access grafts typically do not sustain arterial pressures however is there is impeded outflow then arterial pressures can be realized. A graft with burst pressure of 120mmHg is considered for the purpose of this analysis as a graft that has a potential to fail. Based on the data provided, there are 2.56 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 0.5% that a graft will have the potential to fail in acute aneurysm at 120mmHg.

Suture Retention Strength Test[2]

This test is an evaluation of the ability to suture the graft material to the host tissue. Using a curved surgical needle, a 6-0 Prolene suture is passed through one wall of the graft 2 mm from the free edge using a custom designed fixture. The suture is placed through a location on the graft that is free of any adherent tissue to provide an accurate worst case measure of suture retention strength. The suture is pulled a few inches through the needle hole and the tissue sample is loaded into a tensile tester (Instron Model #5865, Merlin Software Version 5.31) clamping both suture ends in the top wedge grip and then clamping the within the bottom grip. Both ends of the suture are pulled in one direction at 150mm/min with the vessel held stationary. The maximum force that is reached before the suture pulls through the wall of the vessel is taken as the suture pull strength (Attachment A-1.2).

Results

The measured suture retention strength of frozen UVG was on average 3.8N with a standard deviation of 1.3N and a range of 1.8 - 5.4 N. The values all exceeded 1N which the rule of thumb in the industry for acceptable suture retention strength (Attachment A-1.1). Results are shown in Table 3. Data from fresh tissue tested at a different time using a 5-0 suture are included in the data table for comparison.

[1] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

[2] ISO 7198 Section 8.8

Table 3: Suture retention strength fresh tissue and of frozen UVG after thaw and rehydration.		
Sample type	**Suture retention strength (N)**	
	Mean ± SD	**Range**
Fresh Umbilical Vein (n =8)	2.9 ± 1.1	1.3 - 4.4
Processed Frozen Vein (n=10)	3.8 ± 1.3	1.8 - 5.4
SD = standard deviation		

Failure analysis

Graft consistency and the potential for suture pull out during implantation of the UVG graft when stored as frozen graft and implanted as an access graft is addressed using statistical methods below.
Graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The suture retention strength is the best available measure of the likelihood of failure to achieve an suitable anastamoses using the UVG. The data are provided in Table 4.

Table 4: Range and confidence intervals for burst pressure

Lot #	Suture Retention (N)
10746	3.42
10890	2.38
10896	2.39
10911	5.14
10917	5.42
10919	4.26
11029	4.41
11353	3.42
11355	1.79
11376	5.05

Statistic	Result (N)
[3]N	10
Mean	3.77
Lower Confidence limit -95%	2.85
Lower Confidence limit -99.5%	2.27
Min	1.79
Max	5.42
Standard Deviation	1.29

A suture retention strength of 1N is considered to be sufficient to allow for transplantation of the graft without ripping or compromising the graft at the anastamoses. Based on the data provided, there are 2.15 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 1.6% that a surgeon could have difficulty with the implantation due to abnormally low suture retention strength.

Strength After Repeated Puncture[4]

If the graft is used for hemodialysis access the graft will be punctured repeatedly during its use, it is necessary for the tissue to withstand that kind of mechanical insult without forming an aneurysm. The approach taken was to evaluate the strength of ring segments of the LifeCelll UVG when pulled in uniaxial tension to failure (Attachment A-1.2). The tests were performed on

[3] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

[4] ISO 7198 Section 8.3.4

segments containing 24 holes/cm2 (equivalent to 18 months of clinical use based on ISO 7198). This test is designed for synthetic grafts that do not heal and therefore never get stronger. Applying this test to a tissue graft that is designed to heal in, get stronger and remodel and repair needle punctures was used to evaluate a worst case scenario and is really only relevant in the first few weeks of use.

Results

In these tests the focus was on the strength and extension of the vessel wall after puncture. The failure diameters were on average 10.5mm with standard deviation of 0.7mm and a range of 9.9 - 12.1mm. The failure tension was 5.7N/cm on average with a standard deviation of 1.3N/cm and a range of 4.1 - 8.0N/cm. The data show that the strength of punctured grafts on average is greater than 3 times the normal physiologic pressures and failed at non-aneurysmal diameters (Attachment A-1.1). The results are shown in Table 5.

Table 5: Strength after repeated puncture of frozen UVG.

N=10	Mean ± SD	Range
Failure tension (N/cm)	5.7 ± 1.3	4.1 - 8.0
Internal diameter at failure (mm)	10.5 ± 0.7	9.9 - 12.1
Ratio of tension at failure to physiologic tension (120mmHg)*	3.4 ± 0.6	2.6 - 4.2

SD = standard deviation

*The calculation is Ratio= failure tension [N/m] ÷ physiologic tension [N/m],
where failure tension [N/m] = failure tension [N/cm]*100[cm/m] and
physiologic tension [N/m] = 120[mmHg]*133.3[Pa/mmHg]*(Diameter at failure[mm]*0.001[m/mm])}

Failure Analysis

Graft consistency and the potential for repeated puncture-induced aneurysm formation or rupture of the HUV graft when stored as frozen graft is addressed using statistical methods below.
With respect to repeated puncture testing graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The identified requirement for the multi-puncture assessment is that after simulated 18 months of simulated clinical use as per ISO 7198 that the strength of the graft is not compromised (i.e. below physiological requirements).
For the current frozen configuration the strength testing was performed on ring specimens and their diameters were used to calculate the ratio of the failure strength of the punctured graft wall relative to the calculated equivalent physiologic wall tension[5]. This parameter is the best available measure of the likelihood of aneurysm formation due to excessive puncture of a graft without accounting for the tissue's healing response. The data are provided in Table 6.

[5] The calculation is Ratio= failure tension [N/m] ÷ physiologic tension [N/m],
where failure tension [N/m] = failure tension [N/cm]*100[cm/m] and
physiologic tension [N/m] = 120[mmHg]*133.3[Pa/mmHg]*(Diameter at failure[mm]*0.001[m/mm])}

Table 6: Range and confidence intervals for multi-puncture graft failure

Lot #	Ratio of tension at failure to tension at 120mmHg
10746	**3.09**
10890	**2.62**
10896	**4.18**
10911	**3.53**
10917	**2.65**
11029	**3.76**
11353	**4.15**
10746	**3.09**

Statistic	Result (N)
[6]N	8
Mean	3.38
Lower Confidence limit -95%	2.86
Lower Confidence limit -99.5%	2.5
Min	2.62
Max	4.18
Standard Deviation	0.62

A ratio of 1 is considered to have the potential to fail and there are 3.84 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 0.006% that a graft will fail due to multi-puncture alone.

[6] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

Appendix H – Matrix Integrity of Vascular Grafts after -80°C Freeze Thaw

I. Objective

To develop dehydration and rehydration procedures for Glycerol and Ethylene Glycol.

II. Results

2.2 Dehydration Process

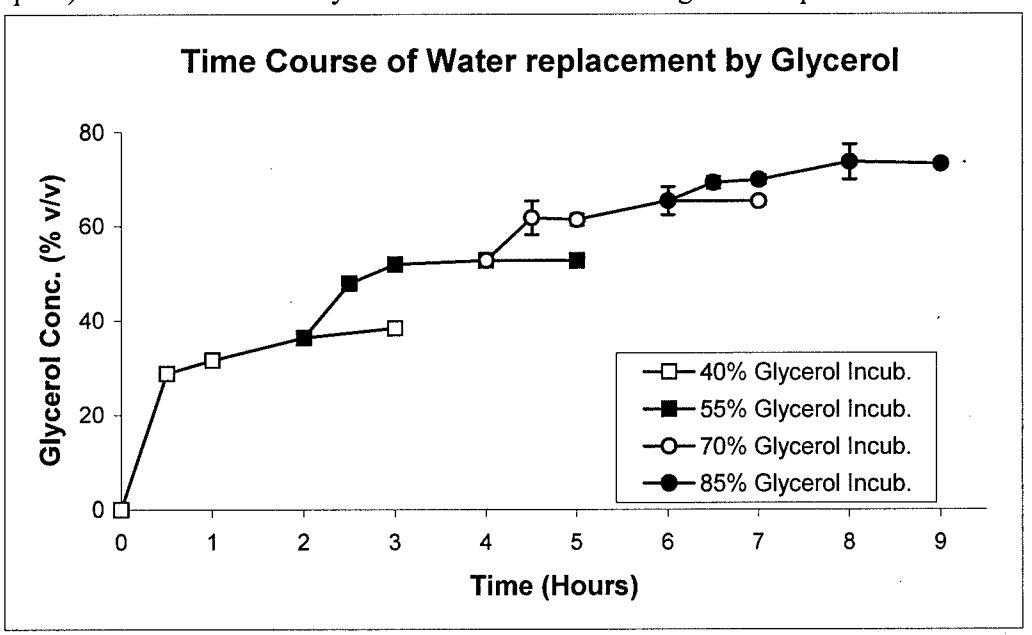
The highest concentration of a solution that would cause the least amount of volumetric change was used to develop the dehydration process. In addition to solution concentrations, the time required for the tissue to reach equilibration at each solution concentration was also determined.

2.2.1 Glycerol (Gly)

Approximately 1 cm pieces of vascular graft were exposed to increasing glycerol (v Gly/v saline) concentrations for two hours at room temperature under constant agitation (85 rpm). The step-wise equilibration was measured using a refractometer (Brix % method).

The equilibration data are shown in Graph 1. The results suggest the following equilibration scheme: 40% v/v Glycerol for 1 hour, 55% for 1 hour, 70% for 1 hour, and 85% for 1 hour. This produces final Glycerol levels of ~73% v/v inside the tissue.

Graph 1) Concentration of Glycerol in tissue matrix during water replacement.

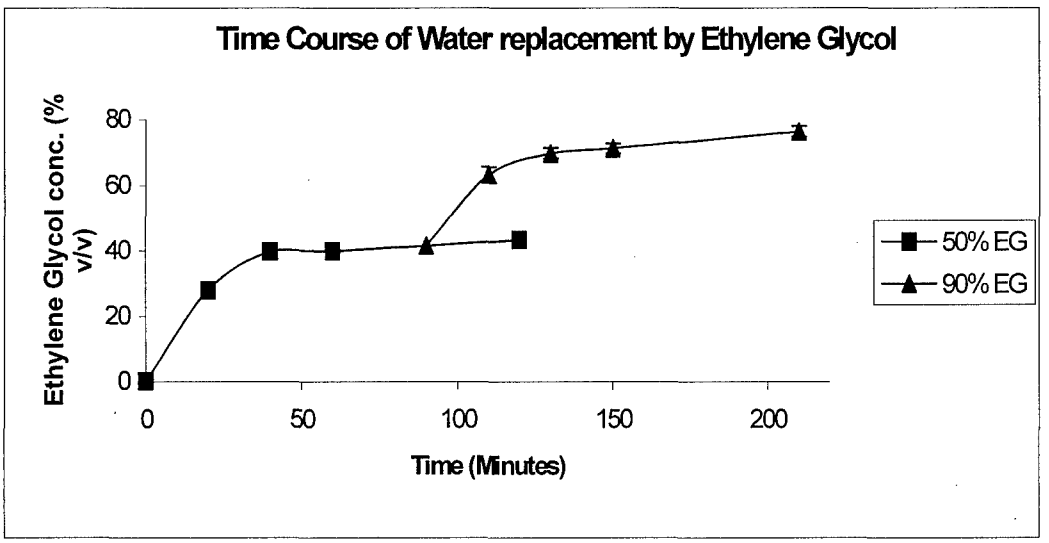


5.2.2 Ethylene Glycol (EG)

Approximately 1 cm pieces of vascular graft were exposed to increasing ethylene glycol (v EG/v saline) concentrations for two hours at room

temperature under constant agitation (85 rpm). The step-wise equilibration was measured using a refractometer (Brix % method).
The equilibration data are shown in Graph 2. The results suggest the following equilibration scheme: 50% v/v EG for 40 minutes and 90% for 40 minutes. This produces final Ethylene Glycol levels of ~71% v/v inside the tissue.

Graph 2) Concentration of Ethylene Glycol in tissue matrix during water replacement.



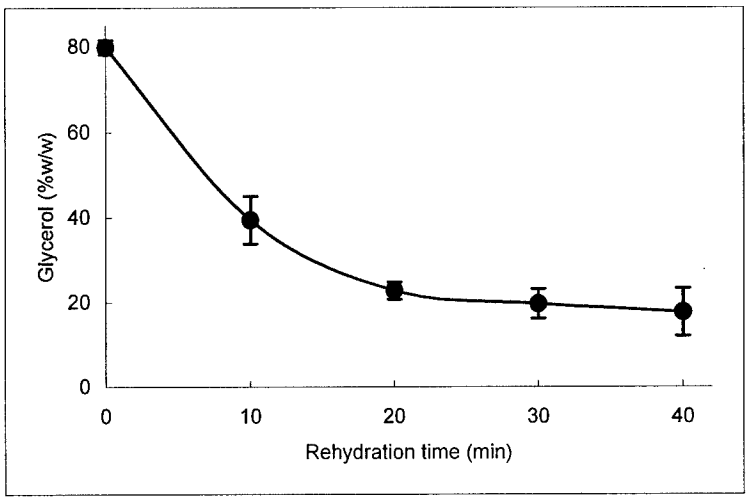
(i) 2.3 Rehydration process

Determined the concentration of Glycerol or Ethylene Glycol remaining in graft after stagnate rehydration in 10 ml saline/cm for different durations.

2.3.1 Glycerol (Gly)

Glycerol concentrations decreased rapidly during the first 20 minutes to ~22% v/v. Then slightly decreased until 40 minutes rehydration where ~18% v/v glycerol remained in the tissue (see graph 3).

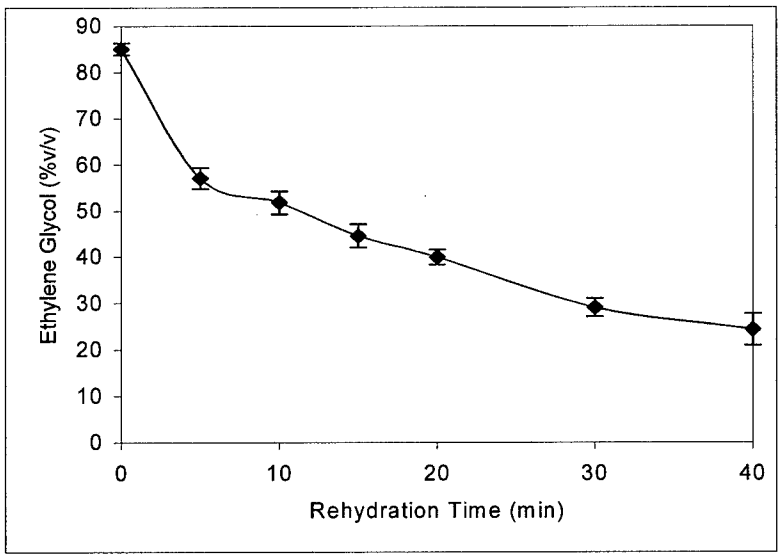
Graph 3) Glycerol Concentration in graft during re-hydration



2.3.2 Ethylene Glycol (EG)

Ethylene Glycol concentrations decreased slower than the glycerol treated grafts and after 40 minutes of rehydration, ~24% v/v ethylene glycol remained in the tissue (see graph 4).

Graph 4) Ethylene Glycol Concentration in graft during rehydration



(ii) 2.4 Residual Moisture in the treated graft

Residual moisture is the water content (% w/w) in the graft after the water replacement treatment.

2.4.1 Glycerol

Residual moisture was determined to be 12.2% w/w.

2.4.2 Ethylene Glycol

Residual moisture was determined to be10.3% w/w.

III. Analysis

For both glycerol and ethylene glycol, each equilibration step can be standardized to a processing time of 1 hour ± 15 minutes. The glycerolization process requires a series of 4 increasing concentrations of 40%, 55%, 70%, and finally 85% while ethylene glycol only requires 2 concentrations of 50% and 90%.
After stagnate rehydration of 40 minutes in 10ml saline/cm on the bench top, samples contained ~18% v/v residual glycerol or ~24% v/v ethylene glycol residual. Finally, the amounts of residual moisture in the samples were 12.2% w/w for glycerol and 10.3% w/w for ethylene glycol.

IV. Discussion

Glycerol and ethylene glycol are both capable of water replacement for preservation of Umbilical Vein Grafts. However, more data on the toxicology profiles of these molecules will be required prior to their introduction into the umbilical vein graft manufacturing process.

V. Conclusions

More testing and research are required to determine the in vitro and in vivo effects of storage in these solutions before a determination is made regarding which solution may be better for preservation of Umbilical Vein Grafts.

Appendix J - In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

I. Objective(s)

To evaluate and compare the histological and mechanical properties of umbilical vein grafts processed either in Glycerol or Ethylene Glycol to those processed and frozen in X-101.

II. Protocol

Three cords (lots 13755, 13581 and 13754) were dissected then divided in 3 equal pieces of ~13-15 cm each and processed through 3rd PBS after DNase as per standard operating procedures (SOP) separately. Each piece was subjected to one of the three treatments.

The X-101 treatment followed SOP with one 4 hour ± 10 minute incubation (~4 hour) followed by a second 4 to 24 hour incubation (~19 hours) of 35% w/w at 10ml/cm each under constant agitation of 100rpm. After processing, X-101 samples were packaged as per SOP in Tyvek and Foil and frozen to -80°C.

The Glycerol treatment consisted of a series of 4 incubations with increasing concentrations of 40%, 55%, 70%, and 85% glycerol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rpm. The Ethylene Glycol treatment consisted of a series of 2 incubations with increasing concentrations of 50% and 90% ethylene glycol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rmp. Glycerol and Ethylene Glycol samples were packaged in 60ml bottles with 10ml/cm from the last incubation solution used (85% for Glycerol and 90% for Ethylene Glycol). Glycerol and ethylene glycol samples remained on the bench top at room temperature until time of testing.

Prior to testing, samples were rinsed as follows:

- X-101 samples received 2 30 minute 100rpm agitation washes with 0.9% sodium chloride at 10mL/cm.
- Both Glycerol and Ethylene Glycol samples received a 40 minute stagnant wash in 0.9% sodium chloride after 5-7 inverts at 10mL/cm.

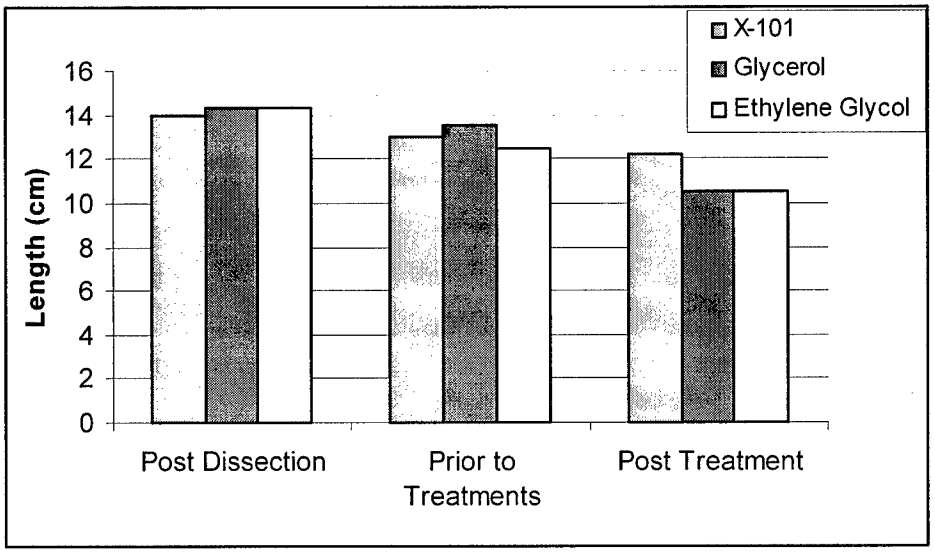
There was one deviation during the performance of the burst test. A burst pressure for the X-101 condition of lot 13755 could not be determined as the test sample kept detaching from the testing apparatus at the higher flow rate.

III. Results/Analysis

Visual Observations

Characteristics such as length, number of coils and lumenal diameter were noted post-dissection, and pre- and post-solution treatments. The length of the samples does seem to decrease following dissection with the processing of the tissue and then again with the loading of the preservation solutions (Graph 1).

Graph 1) Changes in length through dissection and treatments



After the treatments and prior to packaging samples were again observed for macroscopic differences (see Chart 1).

Chart 1) Visual Appearance after Treatments

Sample ID	Treatment	Appearance	Lumen
13754	X-101	Off white	Closed
	Glycerol	Translucent	Closed
	Ethylene Glycol	Off white	Opened
13581	X-101	Off white	Closed
	Glycerol	Translucent	Closed
	Ethylene Glycol	Off white	Opened
13755	X-101	Off white	Opened
	Glycerol	Only Translucent outside WJ	Opened
	Ethylene Glycol	Off white	Opened

Histology

In general, histological examination revealed X-101, Glycerol and Ethylene glycol samples all contained some amount of nuclear debris. X-101 samples contained the least and it was concentrated close to and in the vein wall, as opposed to the Glycerol and Ethylene Glycol where more debris was evident but scattered throughout the Wharton's Jelly. A matrix evaluation of holes, collagen damage and collagen separation/orientation showed Glycerol and Ethylene Glycol samples are comparable to X-101. The basement membrane and internal elastic lamina were present in all samples and no MHC I or II were detected. (See Chart 2 for details and Figures 1-6 for representative photographs)

Chart 2) Histology slide review and scoring

Sample ID	Treatment	Nuclear Debris (Present or Absent)	Holes	Collagen Damage	Collagen Separation/Orientation	Basement Membrane (Present or Absent)	Elastic Lamina (Present or Absent)	MHC I/II (Positive or Negative)
13754	X-101	Present	0	0	0.5	Present	Present	Negative
	Glycerol	Present	1	0	1	Present	Present	Negative
	Ethylene Glycol	Present	1	0.5	0	Present	Present	Negative
13581	X-101	Present	0	0.5	0.5	Present	Present	Negative
	Glycerol	Present	0	1	2	Present	Present	Negative
	Ethylene Glycol	Present	0	0.5	1.5	Present	Present	Negative
13755	X-101	Present	0	0	0	Present	Present	Negative
	Glycerol	Present	1	0	0.5	Present	Present	Negative
	Ethylene Glycol	Present	0.5	0	0.5	Present	Present	Negative

Grading System 0-4		Holes	Collagen Damage	Collagen Separation/ Orientation
	0	None	Intact	No separation
	4	Extreme situation		

The Figures 1-6 below represent sample 13755, focusing on matrix integrity. Note these are representative photographs for all three grafts tested.

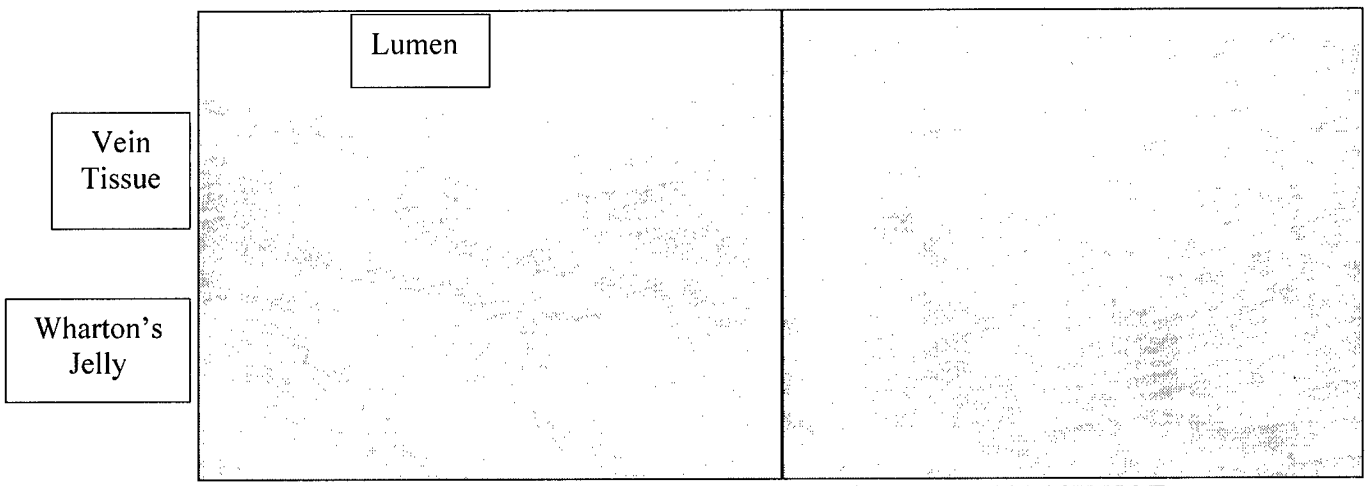


Figure 1) X-101 10X H&E

Figure 2) X-101 20X H&E

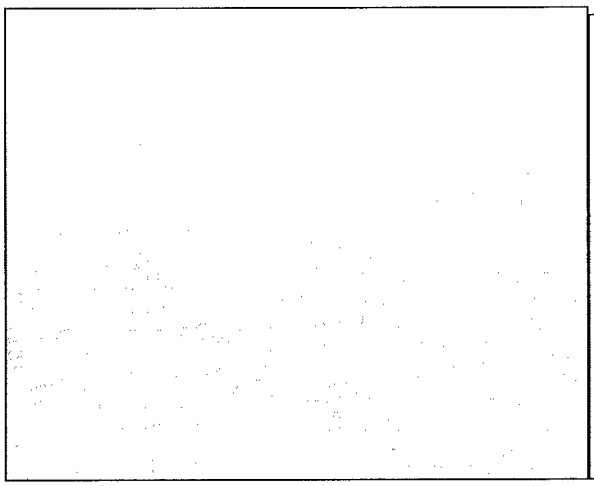


Figure 3) Glycerol 10X H&E

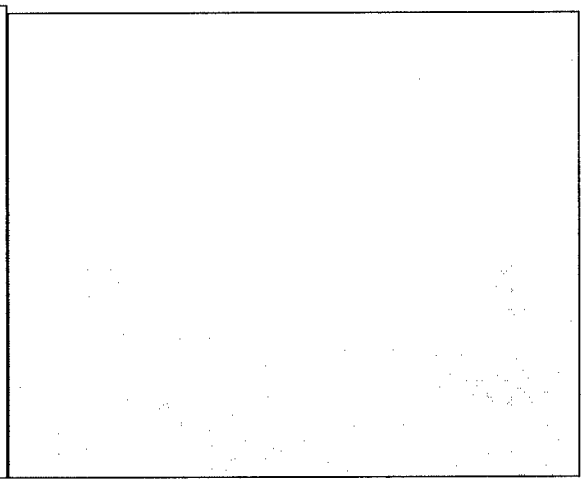


Figure 4) Glycerol 20X H&E

Figure 5) Ethylene Glycol 10X H&E

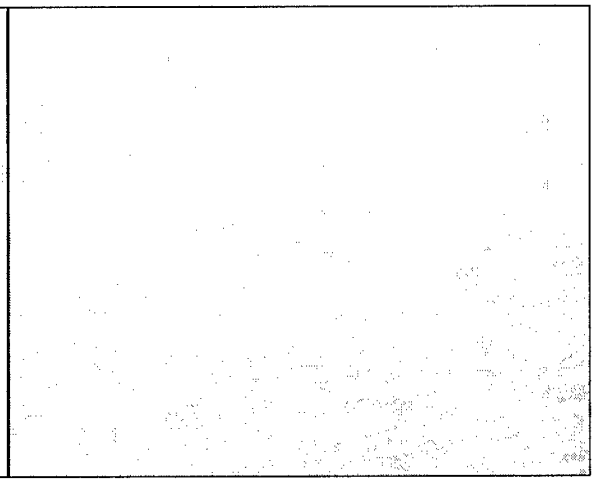


Figure 6) Ethylene Glycol 20X H&E

Mechanical Testing – Statistical Analysis

All mechanical test data were analyzed using a repeated measures ANOVA. This method (similar to a paired t-test) takes advantage of the fact that samples from the same vein graft were treated in different ways for the comparison of those treatment methods. In this way, large variations in the test data that are due to the large variance in donor tissue properties should not hide the effects of the different processing solutions. Statistical significance was considered at $p<0.05$ and a post hoc Neuman-Keuls test was performed where significance was found in the ANOVA.

Circumferential Testing

Circumferential testing simulates the forces that are applied to the walls of the vessel in the circumferential direction when fluid pressure inside the lumen expands the vessel diameter. The testing was conducted in triplicate and the average load at break was used to calculate circumferential tensile strength and the slope of the loading curve was used to calculate a metric of the compliance of the tissue. The raw data from this test are tabulated in Chart 3 (tensile

strength) and Chart 4 (compliance) and the graphs of the ANOVA outputs are in Graphs 2 and 3. Significance in the compliance ANOVA led to a post hoc analysis (Chart 5).

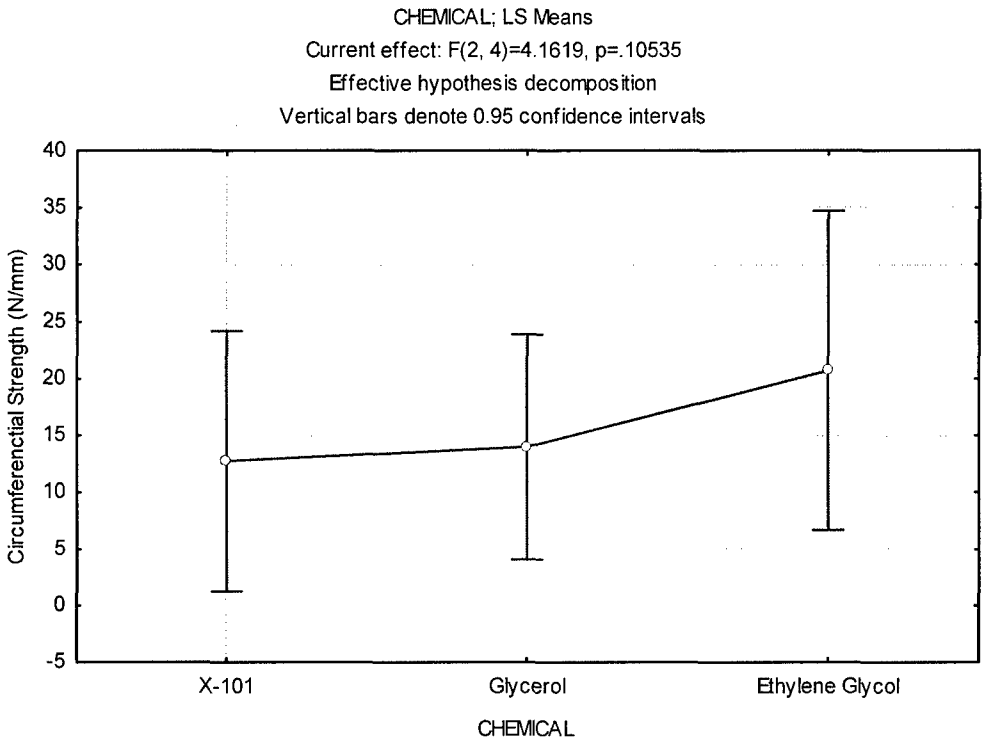
Chart 3) Circumferential tensile strength

		Load @ Break (N)		Circumferential tensile strength (kN/mm)
Sample ID	Treatment	Average	SD	
13754	X-101	7.52	1.40	3.76E-04
	Glycerol	10.01	2.79	5.01E-04
	Ethylene Glycol	15.86	3.07	7.93E-04
13581	X-101	14.09	3.11	7.05E-04
	Glycerol	12.67	0.39	6.34E-04
	Ethylene Glycol	23.50	1.93	1.17E-03
13755	X-101	17.79	2.85	8.90E-04
	Glycerol	19.05	1.51	9.53E-04
	Ethylene Glycol	24.58	5.08	1.23E-03

Chart 4) Circumferential compliance

		Compliance (N/%)	
Sample ID	Treatment	Average	SD
13754	X-101	6.77	0.77
	Glycerol	3.66	1.56
	Ethylene Glycol	3.14	2.26
13581	X-101	6.25	1.54
	Glycerol	5.82	1.11
	Ethylene Glycol	3.63	0.04
13755	X-101	11.49	2.85
	Glycerol	6.71	5.37
	Ethylene Glycol	7.90	2.18

Graph 2) Repeated Measures ANOVA of circumferential tensile strength



Graph 3) Repeated Measures ANOVA of circumferential compliance

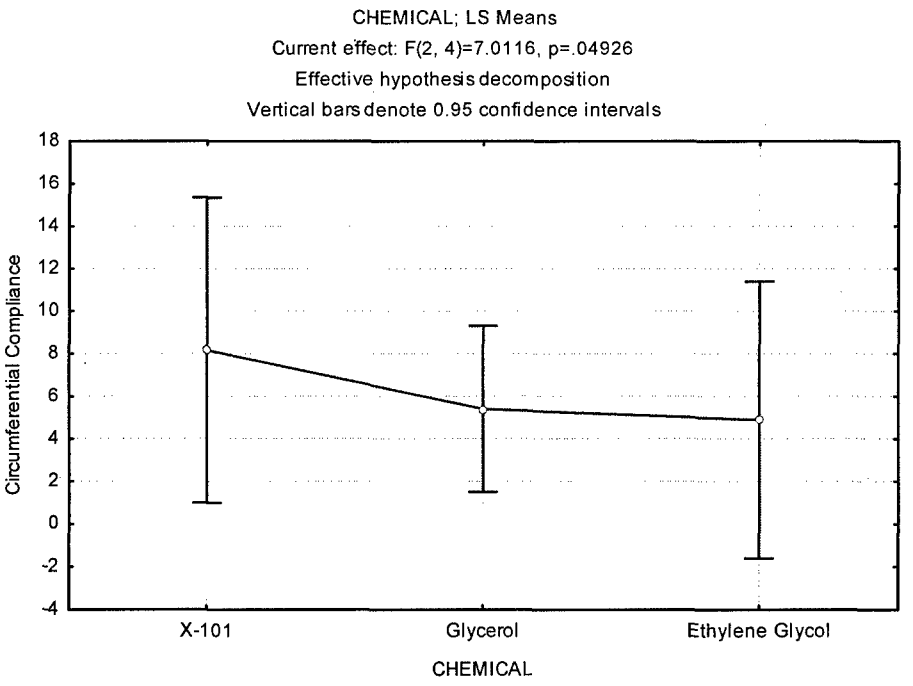


Chart 5) Post-Hoc comparison on circumferential compliance:

Newman-Keuls test; variable - circumferential compliance (RR-0033) Approximate Probabilities for Post Hoc Tests Error: Within MS = 1.3336, df = 4.0000

	CHEMICAL	{1}	{2}	{3}
1	X-101		0.042619	0.053962
2	Glycerol	0.042619		0.617348
3	Ethylene Glycol	0.053962	0.617348	

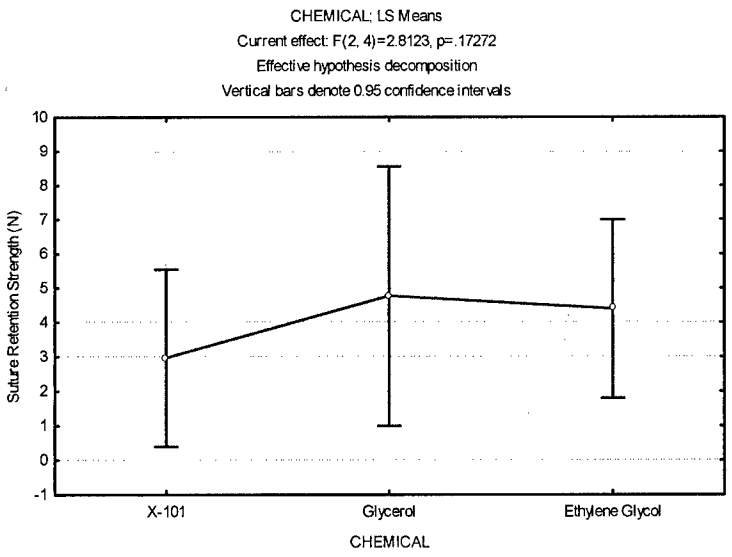
Suture Retention

Suture Retention measures the maximum load (N) it takes for a 5-0 polypropylene suture to tear through the vein graft (Chart 6). Statistically, the maximum load (N) was not differentially affected by the treatments (Graph 4).

Chart 6) Suture retention raw data

Sample ID	Treatment	Maximum Load (N)
13755	Ethylene Glycol	3.71
13755	Glycerol	6.22
13755	X-101	3.95
13581	Ethylene Glycol	5.6
13581	Glycerol	4.92
13581	X-101	3.08
13754	Ethylene Glycol	3.88
13754	Glycerol	3.19
13754	X-101	1.89

Graph 4) Repeated Measures ANOVA of Suture Retention



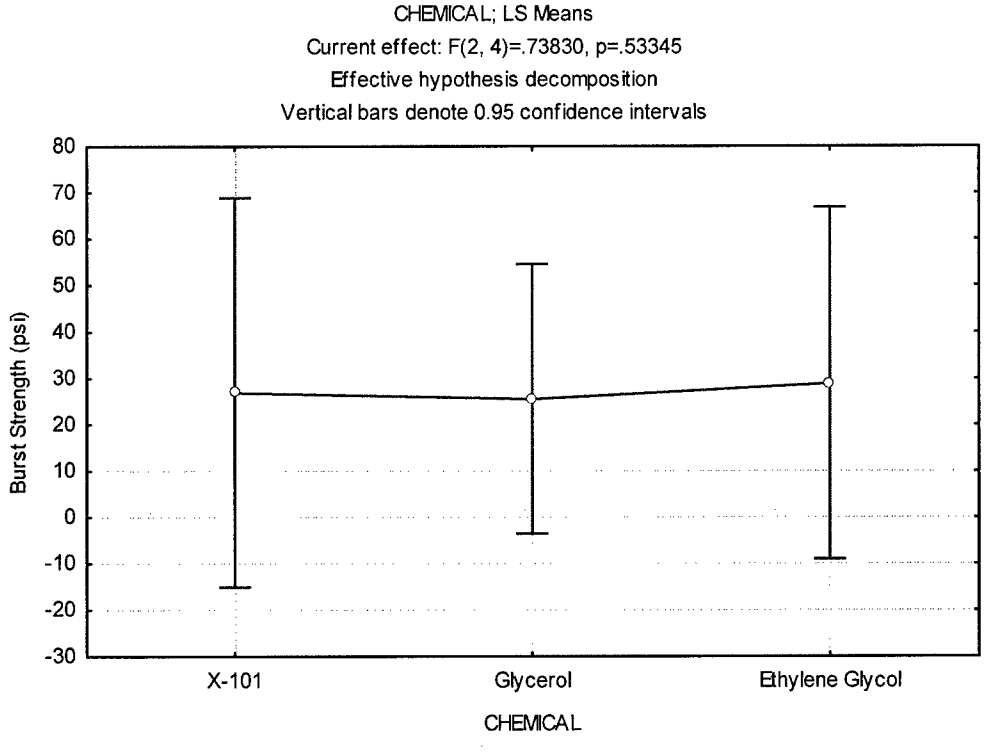
Burst Strength

Burst testing measures the maximum pressure to burst a vein graft. The data are tabulated in Chart 7. Statistically, the burst test data shows no significant difference between preservation solutions (Graph 5).

Chart 7) Burst strength raw data

Cord ID	Treatment	Burst pressure (psi)
13581	X-101	20.8
13581	Glycerol	24.4
13581	Ethylene Glycol	22.4
13754	X-101	13.9
13754	Glycerol	14.4
13754	Ethylene Glycol	17.9
13755	X-101	No data
13755	Glycerol	37.7
13755	Ethylene Glycol	46.3

Graph 5) Repeated Measures ANOVA of Burst strength



IV. Discussion

Visual observations and measurable characteristics demonstrate that the different treatments do seem to have varying effects on the tissue; however, tissue to tissue variability was also observed. Even though the three treatments leave the tissue with a slightly different appearance, upon re-hydration they all look the same.

Histologically, X-101, glycerol and ethylene glycol had varying amounts of nuclear debris. Holes, collagen damage, and collagen separation / orientation for all three sample lots and treatments appear similar. Also for all sample lots and treatments the basement membrane and internal elastic lamina was present. Lastly no MHC I or II was detected on any of the slides.

The only mechanical difference detected between the different treatments was the reduced compliance (N/%) in the Glycerol preserved compared with the X-101 frozen tissue. However, there was very high variability in the test results. Repeated measures ANOVAs were performed to avoid the expected lot to lot variation that is apparent in the data. Still, more analysis would be needed to evaluate the true level of detection that these assays are capable of.

V. Conclusions

Based on the comparison criteria of histology, circumferential, suture retention and burst strength testing the data show that the different processing methods are comparable.

Appendix K - Stability of Glycerol Preserved Human Umbilical Vein Grafts

1.0 Objectives

To determine the stability of umbilical vein grafts (UVG) preserved in glycerol at either 4°C or 20°C after ~1 and 3 months. The stability criteria included sterility, histology, and mechanical properties.

2.0 Materials/Equipment

2.1 0.9% Sodium Chloride solution (saline) (part # 112P0050)
2.2 Glycerol USP grade (Sigma) (part # 112P0022)
2.3 Refrigerator (equipment # 1537)
2.4 Low temperature incubator (equipment # 1508) set to 20°C
2.5 60mL Nalgene bottles
2.6 50mL conical tubes
2.7 Aluminum foil
2.8 7" X 11" foil bags

3.0 Methods

3.1 Three cords were aseptically dissected and processed through the 3rd PBS wash after the DNase step. The tissue was then subjected to the glycerol treatment as follows:

(Note: glycerol solution on %v/v basis with saline.)

- 10mL/cm at room temperature shaking at 85 rpms.
- Incubation with 40% for 1 hour ± 15 minutes.
- Decant the 40% and incubate with 55% for 1 hour ± 15 minutes.
- Decant the 55% and incubate with 70% for 1 hour ± 15 minutes.
- Decant the 70% and incubate with 85% for 1 hour ± 15 minutes.

Samples were packaged with sufficient 85% to cover the sample and placed in either 60mL bottles or 50mL conical tubes depending on the experiment. Samples were shielded from light using foil bags or aluminum foil. Samples were labeled with protocol number, sample ID, storage temperature, and storage time duration.

4.0 Deviations

4.1 Suture retention testing was conducted at a rate of 50mm/min instead of 150mm/min for the 3 month time point (experiment 2), due to operator error. Experiment 238-33, in notebook 238 pages 68-69, was set up to examine the effect between running a suture retention test at a rate of 50mm/min versus 150mm/min. Two vein grafts processed as per SOP, were cut in half and half of each was tested at each speed. The max load (N) strength values were compared using a T-test ($p<0.05$) and even though the 2 speeds did not show significant difference, the samples subjected to the 50mm/min rate were ~23% lower than the 150mm/min samples. The data from the 3 months time point should be considered in light of this finding.

5.0 Results and Analysis

5.1 Data Analysis

Data were analyzed using a repeated measures ANOVA ($p<0.05$).

- Effect of temperatures.
- Effect of time.

- Interaction effect of temperature and time.

Where possible, the data were also compared to the frozen umbilical vein graft process.

5.1.1 Circumferential testing

Circumferential testing simulates the forces that are applied to the walls of the vessel in the circumferential direction when fluid pressure inside the lumen expands the vessel diameter. The data evaluated from this testing includes load at break (N) and compliance (N/%) (Chart 1).

Chart 1) Circumferential testing data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Compliance (N/%)	10608	2.76	9.11	5.18	6.64	6.99	4.26
	13722	4.32	5.70	2.37	9.56	15.09	2.70
	SFH-8	11.92	13.29	6.48	10.57	4.24	6.38
	Frozen	No data available					
Load at break (N)	10608	10.99	18.31	19.70	7.06	9.37	8.88
	13722	11.83	6.83	8.46	17.50	16.80	10.73
	SFH-8	14.12	21.93	22.30	21.79	19.96	19.64
	Frozen	No data available					

Samples from 1 and 3 months and either 4°C or 20°C are comparable to time zero at either temperature for circumferential compliance (N/%) and circumferential load at break (N).

No comparison can be made to the frozen umbilical vein graft as no data were submitted within the IDE.

5.1.2 Multi-puncture testing

Multi-puncture testing uses the same forces as the circumferential test, only the sample is subjected to 24 punctures from a 16 gauge needle. This is to simulate ~18 months of clinical use. The data evaluated here is the load at break (N/cm) (Chart 2).

Chart 2) Multi-Puncture testing data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Load at break (N/cm)	10608	9.11	14.76	3.93	7.07	5.90	2.05
	13722	6.82	5.71	4.60	11.19	6.19	8.34
	SFH-8	10.29	14.02	14.11	11.28	12.64	6.32
	Frozen	5.7 ± 1.3 (n = 10)					

After three months time, samples were comparable for both temperature and time. However, sample 10608 was lower than data from the frozen design for both temperatures at the 3 month time point.

5.1.3 Suture retention strength testing

Suture retention strength measures the maximum load (N) required to pull a 5-0 polypropylene suture through the vein wall. The acceptance criteria for the ability of a vein graft to resist suture tear must be greater than 1N. In addition to acceptance criteria, the data will be used for tracking purposes and comparison over time (Chart 3).

Chart 3) Suture retention strength data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Maximum load (N)	10608	6.84	5.29	2.70	9.16	6.53	3.60
	13722	5.30	7.49	1.61	5.04	3.54	2.41
	SFH-8	4.36	3.08	3.00	6.50	4.45	3.92
	Frozen	> 1					

Samples from 1 month and either 4°C or 20°C were comparable to time zero at either temperature. In contrast, the 3 month samples decline significantly in value compared to both time zero and 1 month samples. This decrease may either be a storage effect and/or partially attributable to the deviation in the test method (see deviation 6.1) Even though it is unknown which factor may have had a greater influence in the decline of the 3 month time points, all values are greater than 1N and meet the acceptance criteria for this test.

5.1.4 Burst strength testing

Burst strength testing measures the pressure required to cause an acute burst of the vein graft. The acute burst pressure must be above 400mmHg to be acceptable (Chart 4).

Chart 4) Burst strength data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Burst strength (mmHg)*	14545				736	1264	1184
	14544				803	1035	1143
	14594				1715	2296	1215
	14587	1858	2021	2639			
	14408	1827	1843	1934			
	14610	939	1569	1934			
	14152	827	1293		652	543	
	SFH-9	1143	1551		1676	1448	
	10577	569	672		564	776	
	Frozen	> 400					

* Data for the test is recorded in pounds per square inch (psi) and converted to mmHg by multiplying the psi value by 51.715.

All values above met the acceptance criteria. Over time there was no effect on the burst pressures at either temperature. (Note: In this experiment, temperature and time could not be evaluated within the same analysis due to the experimental design).

5.1.5 Histological data

Histology was used to evaluate the microscopic characteristics of the tissue. The tissue was subjectively scored on a scale of 1-4, with 1 being the best, in the following categories.

- Holes
- Collagen Damage
- Collagen separation / orientation

In addition to these characteristics, samples were viewed for significant remaining nuclear material and the presence of a basement membrane and internal elastic lamina (Chart 5).

Chart 5) Histology scores

		Temperature					
		4°C			20°C		
	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Nuclear Debris	SFH-18B	Significant	Significant	Significant	Significant	Significant	Significant
	SFH-107	Significant	Not Significant	Significant	Significant	Significant	Significant
	SFH-160	Significant	Significant	Significant	Significant	Significant	Not Significant
Holes	SFH-18B	2.0	1.5	2.5	2.0	3.0	2.5
	SFH-107	1.5	1.5	1.5	1.5	1.5	1.5
	SFH-160	1.5	1.5	2.5	1.5	2.5	2.0
Collagen damage	SFH-18B	2.0	2.0	2.0	2.0	3.0	3.0
	SFH-107	1.5	1.5	2.0	2.0	2.5	3.0
	SFH-160	1.5	3.0	3.0	3.0	3.0	3.0
Collagen separation / orientation	SFH-18B	2.5	2.5	2.0	2.5	2.5	3.5
	SFH-107	1.5	2.0	2.0	1.5	2.0	2.5
	SFH-160	2.5	3.0	3.0	3.0	3.5	3.0
Basement membrane	ALL	Present					
Elastic Lamina	ALL	Present					

NOTE: Scoring was performed by Senior Histologist.

The subjective nature of the histology scoring system, limited the data analysis to qualitative descriptions only. In most cases there was still a significant amount of nuclear debris and in all cases the basement membrane and internal elastic lamina were present. The holes, collagen damage and collagen separation / orientation scores increased from time zero. Also the 4°C samples scored better when compared to the 20°C samples.

5.1.6 Microbiological data

Viromed laboratories conducted standard sterility testing on 1cm segments of tissue using a minimum 600ml media. No growth was the only acceptable result for this test (Chart 6).

Chart 6) Microbiology data

	Temperature	4°C			20°C		
	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Microbiology	SFH-18B	No growth	No growth	Growth (1)	No growth	No growth	Growth (2)
	SFH-107	No growth	No growth	No growth	No growth	No growth	No growth
	SFH-160	No growth	No growth	Growth (3)	No growth	No growth	No growth

1) Coagulase Negative Staphlococcus
2) Propionibacterium Acnes
3) Bacillus Sp. Not Anthracis

The data implies that over time organisms are growing in the tissue; however, this might not be the case. There may be a distribution of organisms along the length of the graft that could account for some samples containing micro-organisms while others will not. In addition, the no growth requirement on the sterility testing was for samples processed in a controlled clean room environment as opposed to a standard laboratory area that the samples were actually processed in.

6.0 Conclusions

6.1 Umbilical vein grafts (UVG) preserved in glycerol stored at either 4°C or 20°C after ~1 and 3 months are comparable to the same UVG stored for 0-2 weeks for the following criteria:

- Circumferential load at break (N)
- Circumferential compliance (N/%)
- Multi-puncture load at break (N)
- Burst strength

6.2 For Suture strength the data collected on UVGs preserved in glycerol stored at either 4°C or 20°C after ~3 months were significantly different from the same UVG stored for 0-2 weeks. However, no conclusions can be drawn due to the deviation in applied loading rate

6.3 No conclusions will be drawn at this time from the histology data because of its subjective nature.

6.4 No conclusions will be drawn at this time from the microbiology data because of the difference in processing environments.

6.5 Based on the data, umbilical vein grafts stored in 85% v/v glycerol solution may be stored at either 4°C or 20°C for up to 3 months.

Appendix L – In Vivo Evaluation of Processed Human Umbilical Vein and Artery in a Small Animal Model (Summary Report)

Objectives:
The following are reworded objectives described by the protocol:

1. Determine if umbilical vessels differ from adult vessels with respect to repopulation of the initially acellular matrix.
2. Determine if vessels processed with Pulmozyme differ from those processed with bovine DNase (bDNase) with respect to repopulation of the initially acellular matrix.
3. Determine if arteries differ from veins, either from adults or umbilical vessels, with respect to repopulation of the initially acellular matrix.

In addition to repopulation (defined as cellular infiltration and infiltration distance) the evaluation of differences is to be extended to:

1. Cell types present
2. Extent of inflammatory response
3. Extent of encapsulation

The following six sample types were implanted in 15 animals, five in each in a randomized way.

1. UAP=umbilical artery (Pulmozyme)
2. UVP=umbilical vein (Pulmozyme)
3. UVB=umbilical vein (bDNase)
4. UAB=umbilical artery (bDNase)
5. AAB=adult carotid artery (bDNase)
6. AVP=adult jugular vein (Pulmozyme)

The evaluation and statistical plan (section 7.0) describes the standard scoring methods used by LifeCell Corporation for this type of study. It includes revascularization and collagen structure, not previously mentioned. Also, it describes cell counting of natural killer cells. The samples are to be randomized and blinded for scoring by an independent investigator (i.e. not in the vascular group). The exact statistical analyses to be performed are not well clarified with respect to the study objectives noted above. (Note: Significance was described in the protocol as a difference of 2.5 on the histological scores and the typical standard deviation was given as 1-1.5. Based on this information an outside statistician evaluated the study design and determined that it had a power > 90%. The values provided were based on summation scores (adding 6 scores for a possible total of 24) and not 0-4 scores and his power analysis was also based on 5 samples not the 6 that were used in the study).

Actual Analyses:
The following categories were scored.

1. Encapsulation
2. Infiltration
3. Infiltration distance
4. Inflammation

5. Revascularization

LifeCell's regular veteran scorer trained another scorer for the first time in evaluation of these types of samples for these types of endpoints and both scorers scored the nude mouse histology slides. The scorers were compared via a multiple t-test (on all of the dependent variables). The t-tests showed significant differences ($p<0.01$) in 2 of the 5 dependent variables. This result prompted a more powerful multivariate 2-way ANOVA comparing the scorer and the sample types and their interaction. Scorer had a statistically significant effect and interacted with sample type. It is assumed that the difference is an indication that the new scorer needs more experience to be able to evaluate histology slides of this kind in this way in a more consistent manner to the experienced scorer. Based on these results only the veteran scorer's scores were used to evaluate the data and only the veteran was asked to score the rat data.

There is a note in the study observation report regarding inflammation of the wound site noted upon harvest of the samples. There is no evidence that this gross inflammation noted for 3 samples was related to the actual samples (they did not have high inflammation scores) and therefore were all included in the above analysis.

To check for animal to animal variations a multivariate analysis of variance was performed on the 15 animals (one way) and the five independent variables above. A similar analysis was done comparing the five different sites (one way). Neither tests demonstrated a significant effect.

Objective 1 (adult vs umbilical vessels):
Due to the subsequent hypotheses implied in objectives 2 and 3 that process and vessel type may affect cell response, like processes and vessels were compared. Thus, two direct comparisons (t-tests) were done: one comparing the adult artery to the umbilical artery both processed with Bovine Dnase (bDNase) and the other comparing the adult vein to the umbilical vein both processed with Pulmozyme. This comparison was done across the five dependent variables for both the nude mouse and rat data.

Results:

Nude Mouse:
The Adult Artery had:

- more cell infiltration
- less inflammation
- less encapsulation

compared to the Umbilical Artery.

The Adult Vein had:

- more revascularization

compared to Umbilical Vein.

Rat:
The Adult Artery had:

- more cell infiltration
- less inflammation
- less revascularization
- less homogeneous infiltration

compared to the Umbilical Artery.

The Adult Vein had:

- more infiltration

compared to Umbilical Vein.

Objectives 2 and 3 (vein compared to artery and comparison of process type):
These objectives were combined in a 2-way ANOVA. Since differences were found in objective one the adult vessels were better not included. Also, once excluded the full factorial analysis could be done on the cord vessels to take full advantage of the data and evaluate any potential interactions between the vessel type and the processing method. The ANOVA was performed independently for each dependent variable and the effect of vessel type (vein or artery) and process method (Pulmozyme or bDNase) and their interaction were evaluated for both the nude mouse and rat data.

Results:

Nude Mouse:
The analysis indicates that there were statistically significant differences in all of the dependent variables. Each one is indicated separately below.

Revascularization:
The Pulmozyme process resulted in an increase in revascularization.

Infiltration distance:
The Pulmozyme process resulted in more homogenous repopulation as defined by infiltration distance. The bDNase was significantly less effective in this regard for the artery than it was for the vein.

Encapsulation:
The vein was less encapsulated than the artery and the Pulmozyme reduced encapsulation compared to bDNase.

Inflammation:
The vein had significantly less inflammation than the artery.

Infiltration:
More fibroblast like cells infiltrated the vein than the artery.

Rat:
The analysis indicates that there were statistically significant differences only for infiltration and inflammation. Each one is indicated separately below.

Inflammation:
The vein had significantly less inflammation than the artery.

Infiltration:
More fibroblast like cells infiltrated the vein than the artery.

Note: These results from the rat data are the same as found for these variables in the nude mouse data.

Additional Objective:
While it was not explicitly stated in the protocol the underlying motivation for this study was to evaluate the proposed Pulmozyme process used on the proposed new source of tissue (umbilical cord) compared to the material used in the proof of principle study (adult artery using the bDNase process). To address this objective a One-way ANOVA was performed on the three relevant groups (UAP, UVP and AAB) with post-hoc Dunnet's test used to compare UAP and UVP to the AAB control where the ANOVA showed significance ($p<0.05$). This ANOVA was performed on each of the dependent variables.

Results:

Nude Mouse:
In no variable were the new vessels found to be worse than the control. The infiltration and infiltration distance were better (lower scores) for both umbilical vessels compared to control. Also, the umbilical artery was significantly more revascularized than the control.

Rat:
Both the infiltration distance and revascularization were better (lower scores) for both umbilical vessels compared to control. The only case (for both rat and mouse) where any scores were statistically worse than the control was the level of inflammation for the umbilical artery.

Conclusions:

The adult vessels were of porcine origin rather than human and of necessity they were from different vessels (i.e. not umbilical but carotid and jugular). Any conclusions regarding the comparison of adult vs umbilical tissue are limited by those unavoidable differences. Still, the data indicated a better response in both the adult artery (infiltration, inflammation and encapsulation) and vein (revascularization) compared with umbilical tissue. The source of the difference can only be speculated, but may be related to the higher surrounding Wharton's Jelly with its significant GAG and HA content. Still, despite the differences all of the scores were relatively low and indicative of tissue that is capable of being infiltrated, revascularized and remodeled by host cells *in vivo*.

The analysis of the effect of cellular removal process on the different umbilical vessels revealed a number of important results.
The process using Pulmozyme resulted in tissue that was significantly more revascularized, less encapsulated and more homogeneously infiltrated.
The umbilical vein incites less inflammation, allows more infiltration, and is less encapsulated compared to the umbilical artery.
Also, while Pulmozyme makes both vessels more homogeneously repopulated it has a more significant impact on the artery.

These results lead to the obvious conclusion that shifting from bDNase to Pulmozyme will only have a positive effect on the matrix. This is possibly due to better cleaning.

The comparisons of the umbilical vessels to the adult artery indicate that the vein graft should perform equivalently. That is, when the vein graft is used clinically as an allograft it is expected to perform as well or better than processed ovine arteries did when they were used as allografts with respect to host cell repopulation, vascularization and lack of inflammation. The artery indicated slightly higher inflammation in the rat but still scored in the midrange of the scale and would be considered an acceptable response in this study.

Appendix M - In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

A. INTRODUCTION

B. A. Purpose:

The objective of the study is to assess the biocompatibility of the test articles relative to the controls. Biocompatibility is indicated by well integrated tissue that is populated by non-inflammatory host cells and supported by neovascularization of the tissue following implant. Based on this criteria, the test articles and control samples were ranked from most biocompatible (a score of 1) to least biocompatible (a score of 4) by the pathologist.

C. B. Justification:

The laboratory rat is an accepted animal model for microsurgery implantation procedures and biocompatibility testing of biologically derived materials and polymers. Surgical methods used to subcutaneously implant a variety of materials in rats are well described in *Experimental and Surgical Technique in the Rat.*[1] A Medline search of the scientific literature indicates the laboratory rat is an accepted animal model for subchronic testing of both polymeric and biologically derived materials.

D. EXPERIMENTAL DESIGN

E. A. General Description:

On Study Day 0 (June 29, 2004), rats were subcutaneously implanted at one of four sites (Figure 1) with one (1) segment from each of two (2) different Test Article umbilical vein graft lots and one (1) segment from each Control Article segment (Table 1). Post surgery weights were recorded twice a week. Rats were observed daily; any abnormalities were recorded.

The study terminated on Study Day 21 (July 20, 2004) all surviving rats were submitted for necropsy. At the time of necropsy surgical implant sites were observed for any abnormalities, implants were removed, photographed, and embedded in paraffin and shipped to Pathology Associates Division of Charles River Laboratories, Worcester, MA, for analysis.

F. B. Group Assignments:

A total of twelve (12) rats were obtained of which ten (10) were used on study. Two (2) additional rats were received to ensure that only healthy rats were placed on study and to replace any animals that reacted adversely to the surgical procedure.

[1] Waynforth and Flecknell, *Experimental and Surgical Technique in the Rat.* 1992.

1. 1) Table 1. Implant Labeling:

Sample	Tissue Type	Storage Conditions
A	Positive control article	-65° to - 90°
B	Negative control article	ambient
C	Test Article – glycerol	4°C ± 1°C
D	Test Article – glycerol	37°C ± 1°C
E	Test Article – ethylene glycol	4°C ± 1°C
F	Test Article – ethylene glycol	37°C ± 1°C

H. B. Anesthesia and Analgesia:

Charles River Laboratories International provided the anesthesia. Rats were not fasted as stated in the original protocol (Deviation W040707A). Each rat received an intraperitoneal (IP) injection of a ketamine, xylazine and saline mixture in accordance with SOP-2517, *Anesthesia of Laboratory Rodents for Contract Research Services.* When rats were completely anesthetized, surgical implantation of the Test and Control Articles began. When necessary, rats received additional injections of ketamine/xylazine anesthesia in order to complete the implantation procedure. While under anesthesia, the rats were observed for signs of hypothermia and kept warm on heating pads where necessary.

I. C. Test and Positive Control Article Preparation:

Test Articles and Positive Control Article were supplied to Charles River Laboratories International by Sponsor.

Approximately ninety (90) minutes before implantation the material was aseptically removed from packaging. The Positive Control Article was rehydrated for approximately one (1) hour at room temperature in 0.9% normal saline (two rinses 30 minutes each with 10 mL saline per cm vein). All Test Articles were rehydrated for approximately ninety (90) minutes at room temperature in 0.9% normal saline (three rinses, 30 minutes each rinse, with 10 mL saline per cm vein). These samples were additionally placed on an orbital shaker for the 30 minute rinse cycle.

Each graft (both Test Article and Positive Control) was prepared for implantation by cutting 4 mm rings off of one end of the graft specimen. Prepared implants were stored in labeled sterile Petri-dishes and moistened with saline until needed for implantation.

Samples were taken for pathological analysis from each Test and Control Article. A 0.5 cm section of each type of implant was placed into labeled cassettes (accession number and tissue type) and placed into 10% neutral buffered formalin (NBF). Implants were stored at ambient temperature in fixative until transferred to Pathology Associates Division of Charles River Laboratories International, Worcester, MA. Specimens were shipped by next-day courier on July 7, 2004 with a chain of custody letter.

J. D. Negative Control Preparation:

Synthetic material was cut into 4 mm rings and soaked in 0.9% normal saline for the same period as outlined above for other implant materials.

K. E. Test and Control Article Administration:

On Study Day 0, each rat received subcutaneous implants of a 4 mm graft segment at four (4) different sites (Figure 1). Surgery was performed on rats in random order. Two (2) samples were implanted, anteriorly; one (1) right and one (1) left of the spinal midline (right front and left front) and two (2) samples were implanted posteriorly; one (1) right and one (1) left of the spinal midline (right hind and left hind). Each rat received one (1) Negative Control, one (1) Positive Control and two (2) Test Article grafts.

Each type of Test Article appeared only once in any given rat. Both the implant location as well as adjacency of the various articles were randomized so that any possible effect of these parameters on results was minimized. Order of rat implantation was also randomized to nullify effects of experimental drift (Table 2).

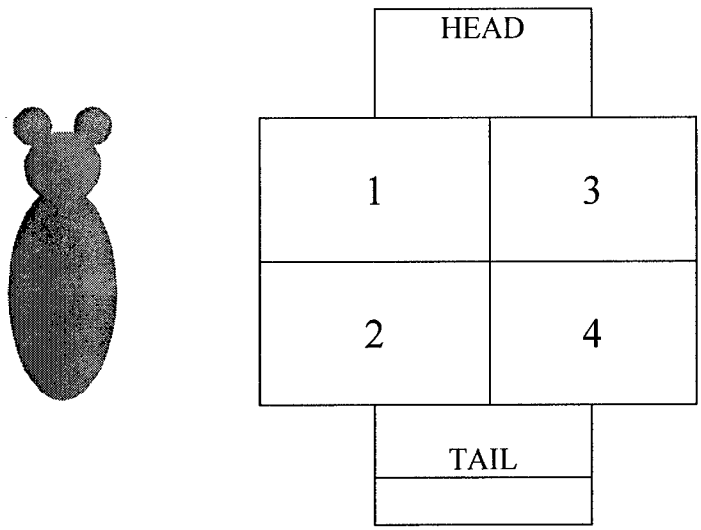
Surgery was performed under aseptic conditions. The surgical site of each rat was wiped with Betadyne and alcohol. A small incision was made with scissors at the implantation site. The subcutaneous tissue and fascia were bisected with scissors, ensuring that there was minimal space around each implant, minimal bleeding and no communication between the four (4) implant sites. The pocket was held open while the article was inserted with forceps. Sutures or wound clips were used to close each pocket. The surgical technique was performed upon all implantation sites in the same manner.

1. 1) Table 2. Implant Schedule:

Rat 1 - #197		Rat 2 - #198		Rat 3 - #190		Rat 4 - #193		Rat 5 - # 195	
A	B	B	A	C	E	F	D	E	A
C	F	F	D	B	A	A	B	B	C

Rat 6 - #185		Rat 7 - #192		Rat 8 - #196		Rat 9 - #191		Rat 10 - #200	
B	E	D	B	A	C	A	F	D	B
D	A	A	F	E	B	B	C	E	A

2. 2) Figure 1. Implant Position Guide:



L. F. Test and Control Article Histopathology (Study Day 0):

The remaining 0.5 cm Test Article and Positive Control samples were submitted for histopathological analysis. The samples were fixed using the appropriate buffers for subsequent hematoxylin and eosin (H&E), Periodic Acid-Schiff's (PAS) and Verhoeff-Van Gieson (Verhoeff) staining. These samples were sent to the Sponsor for evaluation.

M. G. Necropsy:

1. 1) Scheduled Necropsy:

Necropsy was carried out at Charles River Laboratories International Health Monitoring (HM) Department, Wilmington, MA. All rats were submitted for necropsy on Study Day 21 (July 20, 2004).

Euthanasia was performed using CO_2 asphyxiation. No gross examinations were performed except to provide brief comments on any inflammation

abnormalities noted at implant sites. Necropsy was limited to implant collection. Implants were collected one at a time from each rat (4 per rat).

A midline dorsal incision through the skin was made, such that skin was reflected back, and implants in the subcutis were visually observed. Abnormalities associated with the implant sites were documented. The Test Article and any associated encapsulating tissue were dissected free from the subcutis.

A digital image of each implant was taken at the time of Test Article collection. Photos included a label with date of necropsy, study number, animal number, and Test Article implant site information (A-D). Implants were placed into labeled cassettes (accession number, animal number, implant site) and placed into 10% neutral buffered formalin (NBF). One fixative container was used per rat. Implants were stored at ambient temperature in fixative until transferred to Pathology Associates Division of Charles River Laboratories International, Worcester, MA.

N. H. Histology/Histopathology:

All histology and histopathology procedures were performed at Pathology Associates Division of Charles River Laboratories International, Worcester, MA.

The fixed Test and Control Articles and surrounding tissue were appropriately trimmed, embedded in paraffin and several sections obtained that clearly identify the test device/host tissue interface. The resulting slides were stained with hematoxylin and eosin. The samples were read and interpreted by a board-certified veterinary pathologist. The slides were examined by the pathologist in a blinded fashion with no prior knowledge of group assignment for each section. Morphologic parameters that were assessed by light microscopy included, but were not limited to, the following: a) extent of fibrosis/fibrous capsule and inflammation around implant; b) degeneration as determined by changes in tissue morphology; c) number and distribution of the inflammatory cell types, namely polymorphonuclear leucocytes, lymphocytes, plasma cells, eosinophils, macrophages, and/or multinucleated cells; d) presence of necrosis; and e) nature and extent of tissue in-growth into implant (including vascularization).

The pathologist performed a qualitative biocompatibility assessment of the Test Articles and Control Article, including ranked scoring (a score of one (1) representing the most biocompatibility) based upon the following guidelines:

- Less encapsulation is considered more biocompatible
- Less inflammation is considered more biocompatible
- More cell infiltration (non-inflammatory cells) is considered more biocompatible
- Deeper infiltration of host cells (non-inflammatory cells) is considered more biocompatible
- More active vascular channels is considered More biocompatible

O. OBSERVATIONS AND EXAMINATIONS

P. A. Clinical Observations:

Observations of each animal were performed and recorded at least once daily for morbidity, mortality and appearance of the surgical sites. Morbidity would include signs of illness such as, but not limited to, emaciation, dehydration, lethargy, hunched posture, unkempt appearance, dyspnea and urine or fecal staining. All findings were recorded in the study notebook on Charles River *Laboratories Daily Observation Form* (FM-279). A clinical observation summary statement may be found in Section X, Part A.1.

Q. B. Animal Weight Data:

Each animal was weighed on Study Day 0. All animals on study were weighed twice weekly for the duration of the study. Animal weights were recorded on Charles River Laboratories *Animal Weight Record Form* (FM-138). A mean animal weight summary statement may be found on Section X, Part A.2.

R. C. Necropsy Data:

A necropsy summary statement may be found in Section X, Part A.3 of this final report. Copies of IANRs may be found in Section XIII, Appendix, Part C, Attachment 3.

S. D. Pathology Report:

A pathology summary statement is attached.

T. STATISTICAL EVALUATION

Statistical manipulations are not required in this study.

U. RESULTS

V. A. In-Life Measurements and Observations:

1. 1) Clinical Observations Summary Statement:

At the time of animal receipt, animals were clinically observed and were healthy. No unexpected deaths were observed during the study period. There were no detectable abnormalities prior to scheduled euthanasia on Study Day 21 (July 20, 2004).

2. 2) Mean Animal Weight Summary Statement:

At study initiation, mean animal weight was 244.3 grams (g) with individual animal weights ranging from 180 g to 333 g. During the course of the study, mean animal weights progressively increased in all groups. At study termination, mean animal weight was 272.3 g with individual animal weights ranging from 216 g to 337 g.

A mean weight summary table may be found in Section XI, Part C.1 (Table 3), and a chart of mean animal weight measurements in Section XI, Part C.2 Figure 2).

3. 3) Necropsy Summary Statement:

All live animals on study were submitted to Charles River Laboratories Health Monitoring Department for implant collection. At necropsy, visible

implants were detected in all animals surgically implanted with Test Article, with the exception of animal 193 (implant B was not found). Also during the necropsy of animal 198, implant D was cut. Copies of *Individual Animals Necropsy Reports* (IANR; FM-688) may be found in section XIV. Attachment 3.

4. 4) Pathology Summary Statement:

All samples collected at necropsy were sent to PAI for microscopic evaluation. Microscopic findings were graded on a scale from 1-4, (minimal<mild<moderate<marked) according to the intensity and extent of change. A copy of the Pathology Report is attached below.

W. B. Conclusions:

Overall, rats implanted with Test Article appeared normal throughout the duration of the study and at the scheduled necropsy. No animals died unexpectedly on study.

As expected, mean animal weights increased normally as the study progressed.

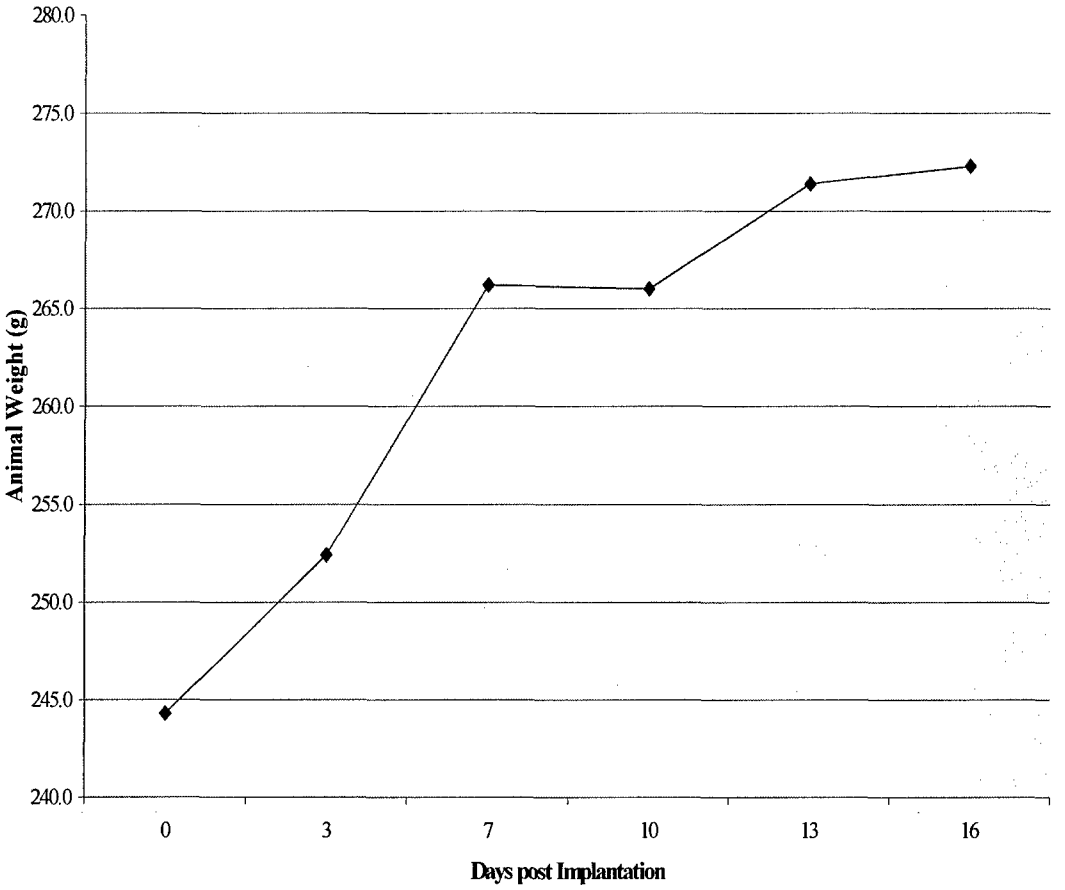
In accordance with the attached Pathology Report, the intensities of alterations varied somewhat between each test article as well as between some test articles and the positive control umbilical vein grafts. Test articles stored at 4° C gave similar response to the positive control, while test articles stored at 37° C had less biocompatibility compared to the positive control.

X. C. Animal Weight Data:

1. 1) Table 3. Mean Animal Weights in Grams ± Standard Deviation (SD):

Group Number	Study Day	0	3	7	10	13	16
	Date	6/29/2004	7/2/2004	7/6/2004	7/9/2004	7/12/2004	7/15/2004
N/A	Mean	244.3	252.4	266.2	266.0	271.4	272.3
	SD	42.8	38.1	35.2	28.2	28.7	33.8

2. 2) Figure 2. Mean Animal Weights in Grams:



Rats were weighed individually twice a week for the duration of the study. Data represented graphically as the average animal weight in grams (g) by group.

Appendix N - In Vivo Assessment of Umbilical Artery Graft in a Porcine Model – Feasibility Study (Summary Report)

EXECUTIVE SUMMARY

The objective of this study was to evaluate human umbilical arteries for applications suited to a small diameter graft. Umbilical arteries were evaluated for the capability to withstand a high pressure, high flow situation for a relatively short period of time (7 days), as well as to assess its resistance to occlusion and aneurysmal dilatation.

Two de-cellularized and vitrified human umbilical artery grafts, obtained from two distinct umbilical cords, were implanted bilaterally as common carotid interposition grafts in one pig. The umbilical arteries handled like well-behaved biological grafts with no gross bleeding or degeneration. The animal appeared to be doing well until day six when the pig was found dead by the animal caretaker.

The necropsy revealed that one of the vitrified human umbilical arteries ruptured; the other underwent a less catastrophic failure that resulted in a hemodynamic leak. Both grafts appeared patent, and neither graft became dilated.

OBJECTIVE

Acellular umbilical cord veins have been developed for use as vascular shunts, but the vein's diameter limits the applications. Methods for producing acellular vascular shunts using umbilical cord arteries will dramatically expand the application of vascular shunts. For example, the umbilical cord arteries are much more suitable for bypass surgery in patients with coronary artery disease: a disease that affects approximately 12 million people in the United States.

RESULTS

Two decellularized and vitrified human umbilical artery grafts, obtained from two distinct umbilical cords, were implanted bilaterally as common carotid interposition grafts in one pig. The grafts were thawed in PBS (pH 7.4) immediately prior to surgery, and photographed with a filled lumen (Figure 1).

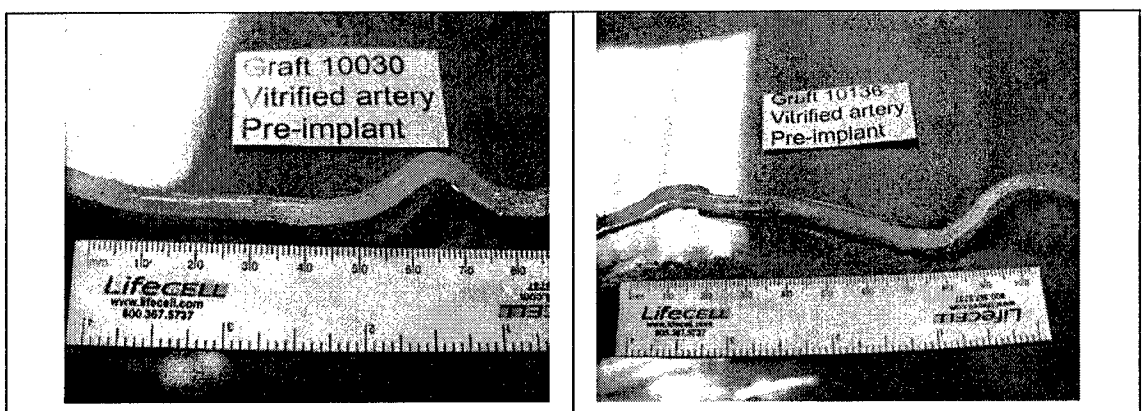


Figure 1: Photographs of umbilical arteries prior to implantation.

Umbilical cord artery 10030A-1 was grafted to the pig's left carotid artery, end-to-side, to generate both the proximal and distal anastomosis, while artery 1036A-1 was similarly grafted to the pig's right carotid artery (Figure 2).

Figure 2: Umbilical artery immediately after implantation as carotid interpositional grafts in a porcine model.

Flow rates were measured at two times following surgery. The left carotid graft flow rate initially measured 40 ml/min, and sixty minutes later measured 200 ml/min. The right carotid graft flow rate was 35 ml/min initially, and at 85 ml/min thirty minutes later.

The pig was observed daily and appeared well with no complication until day 6 when the animal died shortly before noon. Examination of the animal revealed that both sides of the neck were swollen (Figure 3)

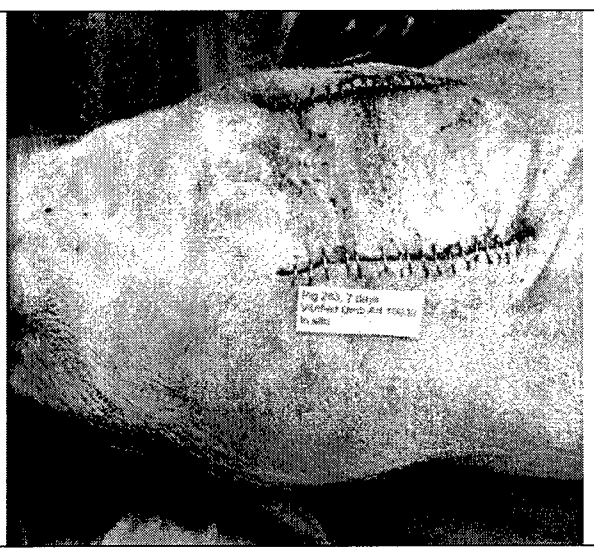


Figure 3: Gross photo of swollen neck at implant site post-mortem

Necropsy revealed large bilateral hematomas. Rupture clearly occurred of the umbilical artery 10136A-1 above the proximal anastomosis of the right carotid artery graft. Graft tissue remained attached to the anastomosis--indicating that the failure was not the anastomosis itself. No gross rupture of the umbilical artery 10030A-1 grafted to the left carotid artery occurred, but a large hematoma was evident (Figure 4).

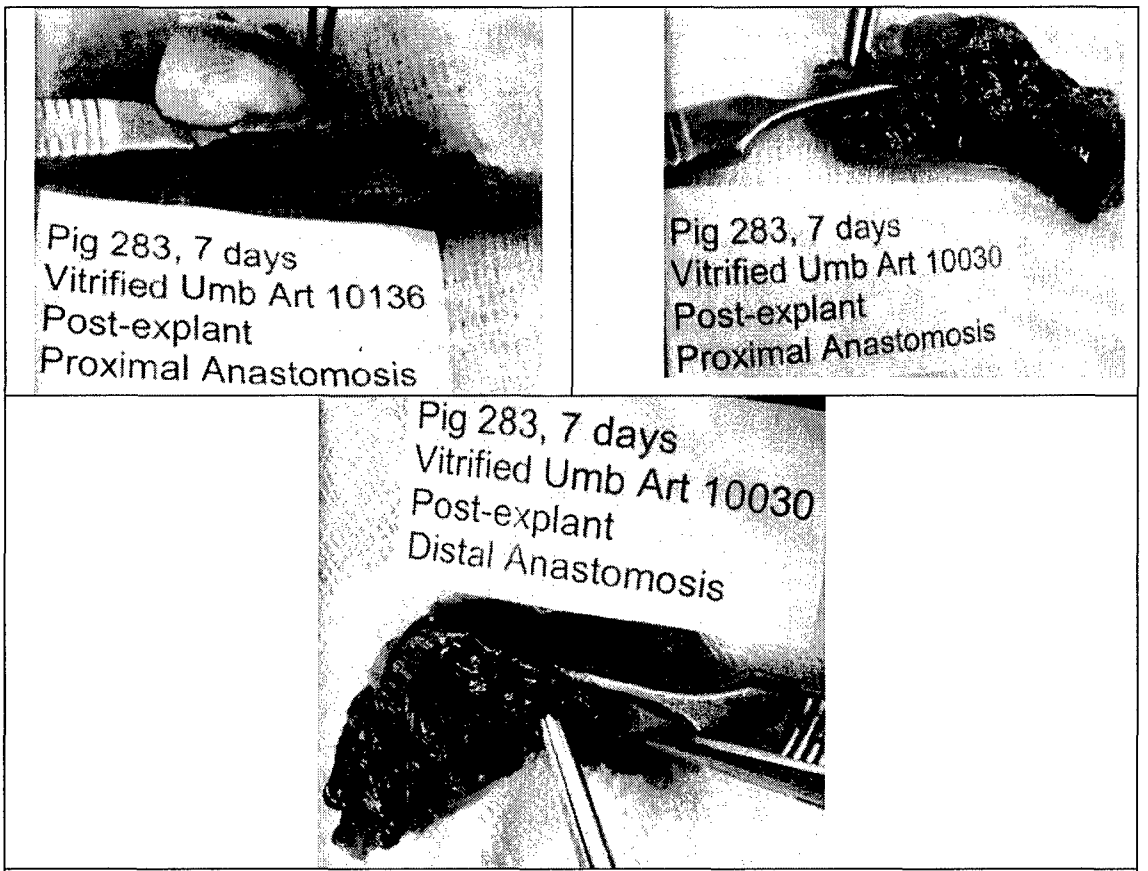


Figure 4: Gross photos of umbilical artery explantation

Gas bubbles next to the left carotid graft were noted (Figure 5). An infectious agent could be the source of the gas bubbles, however, both grafts had negative culture results prior to implant.

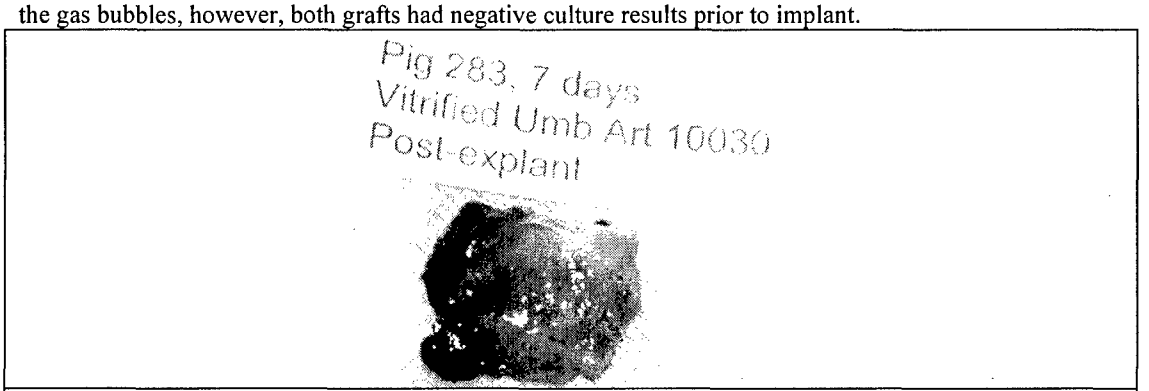


Figure 5: Gas bubbles documented on explanted tissue possibly due to infection.

Both grafts contained loose unorganized clot, and a probe easily passed into each graft. This supports that both grafts were patent. Additionally, no dilation was evident.

CONCLUSION

It is not clear what cause the failure of the grafts in this study. More rigorous evaluation of umbilical artery tissue is warranted.

Appendix O - Evaluation of Umbilical Artery Tissue Dissection

Objective

Observations about arteries that shred, break, and leak.

Data/Discussion

Veins from the umbilical cords were dissected in the tissue lab and used for experimentation. The arteries were further dissected from the amnion using the blunt dissection method. The table below documents the ability to recover acceptable lengths or arterial tissue. Arteries that have blood clots or blood related staining of the tissue are noted. Breakage and leaking of the grafts either during dissection or filling the graft after dissection are recorded as well. There is a full photographic record. Figure 1 shows an example of pre-dissection umbilical arteries with adherent tissue and dissected umbilical arteries. Table 1 catalogues the % of breaks and leaks in unstained sections of artery and stained sections of artery.

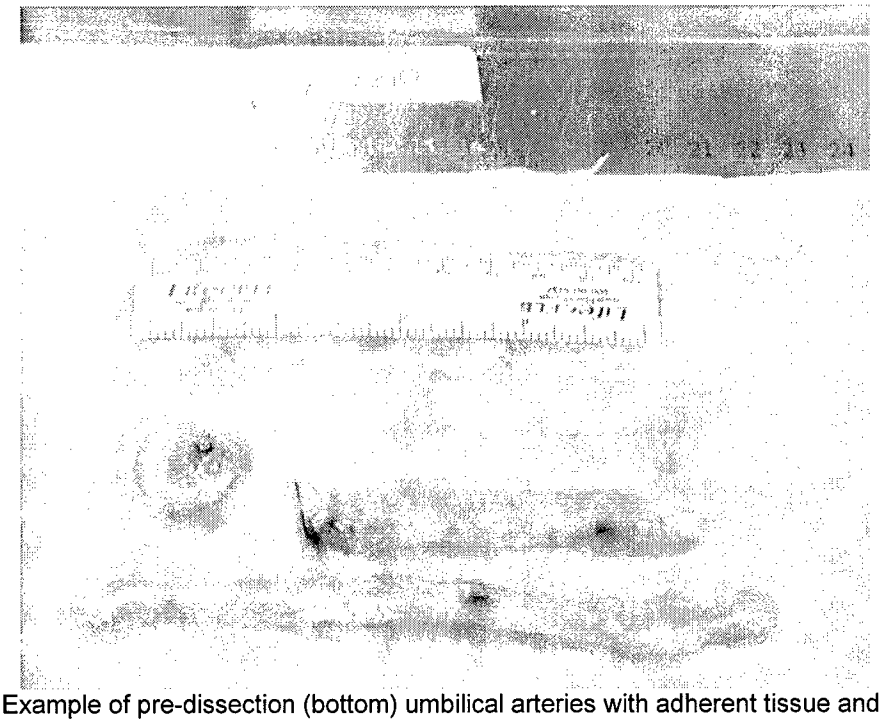


Figure 1: Example of pre-dissection (bottom) umbilical arteries with adherent tissue and following dissection (top).

Example:
12018 A_1 had two breaks total. One occurred at a clot, one in a stained area, and both breaks involved clean areas, therefore, one break occurred at a clot in a clean area, and the other occurred at the interface of a clean and stained area. 12018 A_2 had three breaks total: two at clots (actually between the four clots) and one in a clean area.

Appendix O - Evaluation of Umbilical Artery Tissue Dissection

		Clots		Unstained Portions		Stained Portions		Kinks		Total
Cord #	Cord length	#	break/ Leak	%	Break/ leak	%	break/ leak	#	break/leak	break/ leak
11972 A1	42 cm	0	0	5%C	0	95%C	0	0	0	**0**
11972 A2		0	0	40%V	0	35%V/25 %C	0	0	0	**0**
11984 A1	30 cm	0	0	10% C	0	90% C	0	1	1	**1**
11984 A2		0	0	10% C	0	90% C	0		1	**1**
11810 A1	48 cm	2	1	39%C	1	61%C	0	0	0	**1**
11810 A2		2	0	30%C	0	70% C	0	1	0	**0**
11811 A1	55 cm	TNTC	5	0%	0	100% C	5	0	0	**5**
11811 A2		0	0	0%	0	100% C	TNTC last 20 cm	1	0	**TNTC**
12040 A1	38 cm	0	0	50%V	0	50%V	0	0	0	**0**
12040 A2		0	0	83%C	2	17%C	1	0	0	**2**
12017 A1	57 cm	TNTC	TNTC			100% C	TNTC	0	0	**TNTC**
12017 A2		TNTC	TNTC			100% C	TNTC	0	0	**TNTC**
11948 A1	33 cm	10	2	3%C	1	97%C	2	0	0	**2**
11948 A2		TNTC	2	14%V	1	86%V	2	0	0	**3**
11971 A1	48 cm	TNTC	TNTC			100% C	TNTC		TNTC	**TNTC**
11971 A2		TNTC	TNTC			100% C	TNTC		TNTC	**TNTC**
12018 A1	36 cm	1	1	20%C 40%V	2	40%V	1	0	0	**2**
12018 A2		4	2	50%V	1	50%V		0	0	**3**
12109 A1*	16 cm	1		100%C	1			0	1	**1**
12109 A2*		1		100%C	1			0	1	**1**

Analysis

- Arteries tend to shred upon dissection in areas where they appear dried out, collapsed, and difficult to see due to lack of blood. 10 breaks were counted in the clean areas. Of these, 5 were in clean stretches, 3 were at clots in the middle of clean stretches and 2 were at the interface where clean areas met stained areas.

- Arteries that are extremely clotted, with clots too numerous to count, also tend to shred at multiple points along the vessel. Six arteries with clots too numerous to count correspondingly tore, broke or shred frequently during dissection. Seven arteries with 18 clots sustained breaks at the clots in 6 instances. As mentioned above, three were located in clean areas, and three of those were between clots that were very close together.

- Any large anatomical disturbance, like a "sausage link" kink in the cord, or an unusual twist, has greater potential to break at that point. In one such instance, a cord had looped back on itself. One artery passed through the loop but broke at dissection. The other traveled around the loop, came off in one piece, but later leaked at that point when perfused. Similarly, an extremely bulgy looking clot – wider than 3 mm – may have greater potential to break at that point just before or after the clot. In one instance, breaks occurred on either side of a very large (4mm diameter) clot.

- Finally, arteries that were stained, but not clotty, seemed to hold together. Five arteries with at least 50% consistent staining, but no clots, did not break. Two others with a variegated pattern, but at least 50% stained, also did not break.

Although not currently standard procedure, arteries were perfused with PBS after dissection to check for leaks. There were three instances were arteries that came off in one piece and looked clean would leak, while variegated arteries held the fluid. **It does not seem at this point that it is a simple matter to judge the competence of the artery by color or successful dissection.**

A fresh 15cm length of cord #12109 was dissected to note the differences. The blood was a brighter red and flowed more freely in contrast to frozen/thawed arteries. The arteries looked fairly clean with just a few clots. However, while both 15 cm+ lengths of artery came off the vein in one piece, both broke upon further dissection. Afterward, the blood flushed easily from the artery's lumen when perfused with PBS, and didn't leave a stain, except for a small spot where a clot had been. These pieces did not leak when perfused. Perhaps the fact that the red blood cells had not been lysed by freezing the staining components, Hemoglobin and/or Heme, remained in the red blood cell. See figure 1, which shows dissected, flushed arteries at top, arteries in the amnion pedicle at the bottom, and a cross-section of whole cord at left.

Of the ten cords dissected and the potential for 20 grafts. After breakage the only long vessels left were 6 grafts varying in length from 28 cm to 41 cm but they varied in coloration. Only one 15 cm stretch was really clean a yield of about 2% based on length assessment. These cords were all received from NDRI. It is anecdotal that the cords received from SFH are typically less bloody and clotty than the cords received from Saint Francis Hospital.

Conclusions

Fresh or frozen/thawed arteries did not demonstrate much difference in their ability to hold together during dissection. However, the ease of perfusing the fresh artery and the clean state achieved afterward warrants further investigation into this process. It may be feasible to flush the lumens of the artery at the first holding step stage. If so, this may yield a cleaner arterial graft later.

Appendix P - Porcine Umbilical Vein Acute Implantation Study (Summary Report)

Executive Summary:

The main objectives of this study were to place a human umbilical vein decellularized, cryopreserved, and freeze-dried by the LifeCell method in a porcine model in a physiologically relevant arterio-venous shunt position (carotid artery to jugular vein) and ascertain handling characteristics including tunneling and suturing. Due to the necessity to access these grafts for hemodialysis in their eventual clinical application and due to the wide variation in umbilical vein geometry with respect to coiling, acute puncturability as well as hemostatic properties of the grafts was also assessed. In addition, patency, kinking, uniformity, and flow through the graft were evaluated both prior to and following puncture via angiography and flow probe. The ease of tunneling each successive graft increased with surgeon experience, and each of the grafts sutured well without needle hole bleeding. Each graft had a palpable thrill beneath the subcutaneous tissue of the neck and supported flows of between 250 and 550 mL/min, with the less coiled grafts supporting higher flow rates than more coiled grafts. Grafts were deemed easier to puncture than standard PTFE grafts and comparable to native arterio-venous fistulas. Following puncture, 5 minutes of direct skin pressure was required to control bleeding of the grafts through the skin, and only minor bleeding was observed when the skin overlying the graft was opened. Only one of the four grafts tested remained patent. Of the three that thrombosed, however, two were evaluated in an animal not adequately heparinized and the other, the most coiled used in the study, was believed to have kinked within the subcutaneous tunnel or been traumatized by manipulation of the proximal carotid artery during angiography. Very little thrombus was associated with the patent graft.

Objectives:

The overall goal of this study was to assess the functionality of a decellularized, cryopreserved, and freeze-dried human umbilical vein graft as a hemodialysis access graft. The criteria evaluated included handling of the graft, including suturability and subcutaneous tunneling of the graft. Ease of graft puncture with a 16G hemodialysis needle, time to hemostasis, and evidence of graft wall damage following puncture were also assessed. Finally, patency and flow through a spiraled graft placed in a subcutaneous tunnel was examined.

Results:

A tabular display of the results collected during execution of the study can be found in Table 1. Of the four grafts implanted, all were judged to suture well without needle hole bleeding, due to the hemostatic nature of Wharton's jelly surrounding the vein graft. There were challenges associated with tunneling of the graft related both to its fragility and extent of coiling, with very coiled grafts being more difficult to tunnel. Once implanted, each of the four grafts, regardless of extent of coiling (Figure 1) exhibited a palpable "thrill," or high to low pressure drop typical of arterio-venous shunts. Flow through 3 of the 4 grafts was maintained at greater than or equal to 200 mL/min for the duration of the study. The remaining graft, the most

coiled of the four, became occluded prior to any punctures being performed. The extreme coiling of this graft may have caused its occlusion, but multiple manipulations of the proximal carotid artery during angiogram may have contributed as well. See Figure 2 for angiograms of the implanted grafts. Of the three grafts assessed for puncturability, all were deemed easier to access than conventional synthetic PTFE grafts, had hemostasis achieved with skin pressure in less than 5 minutes, and exhibited only minor bleeding with opening of the subdermal tunnel (Figure 3). One particular graft was initially difficult to access because it was placed partially beneath the muscle layers of the neck and had a hard-to-access configuration over the sternum. Each subsequent puncture of this graft, however, was performed without difficulty. Only one of the grafts remained patent through explant (Figure 4). The two remaining grafts were explanted from an animal not receiving the required final dose of heparin pre-explant.

Animal	Cord ID	Number of Coils	Length	Uniformity	Position	Ease of tunneling	Ease of suturing	Needle hole bleeding	Palpation	Flow	Ease of puncture	Time to superficial hemostasis (skin puncture site)	Hematoma following puncture	Angio-gram post-puncture	Final heparin given?	Thrombus
160	9267	3	18 cm	uniform	Cephlad; left carotid to right external jugular	Difficult to load into 28 Fr tunnel, graft tore	Sutured well	None. Hemostatic	Good thrill	400-500 mL/min → 200 mL/min once 9260 opened to flow	Easier than PTFE	≤5 min	Minimal blood deep in neck only	N/A	None	Clots evident
160	9260	4	18.5 cm	uniform	Caudal; right carotid to left external jugular	Not easy, but improved	Sutured well	None. Hemostatic	Good thrill	250-300 mL/min	Easier than PTFE	≤5 min	Minor bleeding when skin re-opened	N/A	None	Clots evident
161	9391	7	23 cm	uniform	Cephlad; right carotid to left external jugular	Difficult because of tight coil	Sutured well	None. Hemostatic	Good thrill, decreased over time	280-380 mL/min → 150 mL/min → 0	N/A	N/A	N/A	N/A	N/A	Occluded
161	9291	2	23 cm	uniform	Caudal; left carotid to right external jugular	Good tunnel, best for this study	Sutured well	None. Hemostatic	Great thrill	550 mL/min	Easier than PTFE	≤5 min	Minor bleeding when skin re-opened	Patent	Yes	No clots evident, patent on angio-gram

Table 1. Summary of all data collected during protocol execution.

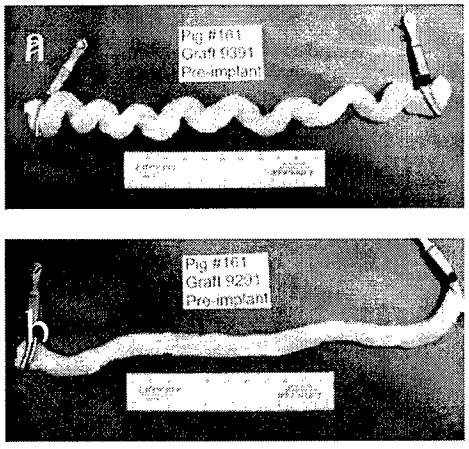


Figure 1. Representative photos of (a) very coiled and (b) less coiled grafts pre-implant.

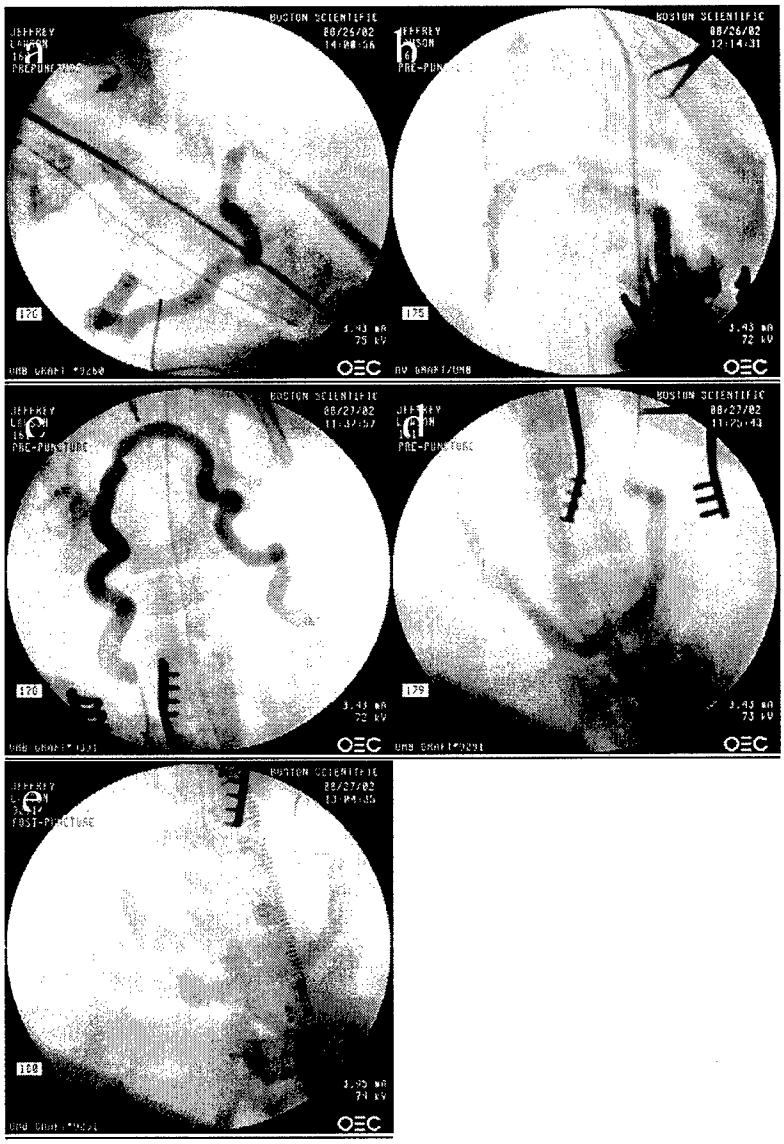


Figure 2. Angiograms of umbilical vein grafts in vivo both (a-d) preceding and (e) following puncture.

(a - graft 9260, b – graft 9267, c – graft 9391, d-e – graft 9291 pre and post-puncture)

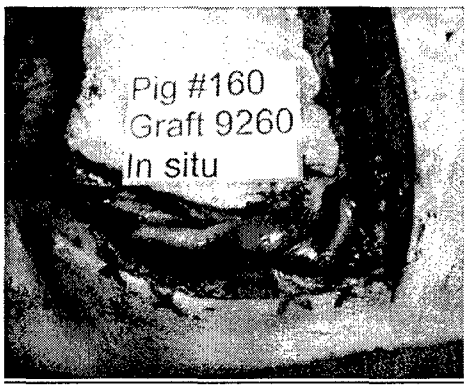


Figure 3. Representative photo of graft lying in opened tunnel following puncture. Xs on the skin mark the puncture sites.

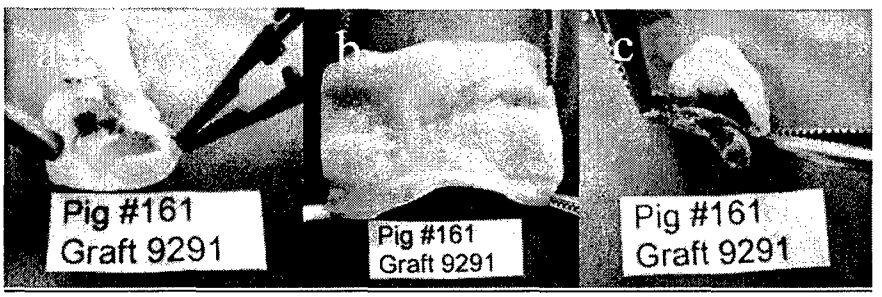


Figure 4. Explanted patent graft 9291. (a) arterial anastomosis, (b) longitudinally opened mid-graft, (c) venous anastomosis.

Histological observations:

Graft 9267 (Pig 160)

Both arterial and venous anastomoses showed good approximation between the native vessels and vein graft. The arterial anastomosis was free of suture line inflammation within the native carotid artery. A suspected cross-species immune response was indicated by complete isolation of the graft at the lumenal surface (Figure 5). No inflammation was observed within Wharton's jelly.

The venous anastomosis was characterized by scattered leukocytes within the outer layers of Wharton's jelly, hemorrhage into Wharton's jelly, and inflammation most likely due to a cross-species immune response not only surrounding the graft lumen but within the native vein as well.

Some deposited material, assumed to be fibrin or the product of opsonization, could be seen at the lumen of the graft.

Punctures were difficult to locate and could not be positively identified. One suspect puncture site was accompanied by a large hematoma within Wharton's jelly.

Sections taken near all 4 punctures sites revealed slight to moderate hematoma within Wharton's jelly, but not the vein wall.

Figure 5. Representative histological section of mounting inflammatory response surrounding graft lumen.

Figure 6. Hematoma near a suspected puncture site.

Approximation of the graft and native tissue was not evident at either the arterial or venous anastomoses as these areas of the tissue sections appeared fragmented. The arterial anastomosis was free of suture line inflammation within the native carotid artery. A suspected cross-species immune response was indicated by complete isolation of the graft by inflammation at the lumenal surface. A deposited layer believed to be fibrin lined the graft lumen which appeared occluded by thrombus. Slight inflamation as well as some hematoma was observed within the relatively small amount of Wharton's jelly in this graft.

The venous anastomosis was characterized by very little inflammation within the outer layers of Wharton's jelly, hemorrhage into Wharton's jelly, and a suspected cross-species immune response not only surrounding the graft lumen but possibly within the native vein as well.

A relatively large amount of deposited material could be seen overlying the cellular immune response to the graft lumen. A significant amount of thrombus was present within the lumen at this anastomosis as well.

No response to suture was evident.

Punctures were difficult to locate and could not be positively identified. One suspect puncture site was accompanied by a large hematoma into Wharton's jelly. Sections taken near all 4 punctures sites revealed slight to moderate hematoma within Wharton's jelly, with the final puncture appearing to cause hematoma within the graft vein wall as well.

Graft 9391 (Pig 161)

Neither the arterial nor venous anastomoses showed good approximation between the native vessels and vein graft. The arterial anastomosis was free of suture line inflammation within the native carotid artery. A suspected cross-species immune response was indicated by complete isolation of the graft at the lumenal surface by inflamation, which was overlaid by proteinaceous deposit and thrombus. The vein wall itself was invaded by some inflammatory cells at an isolated area where there was separation of the vein wall. Slight inflammation was observed at the outer layers of Wharton's jelly and within a relatively large hematoma within Wharton's jelly nearer the vein wall.

The venous anastomosis exhibited no inflammatory response to Wharton's jelly nor hematoma within either the vein wall or surrounding Wharton's jelly. A far lass severe response to the graft lumen was observed, and the native vein looked fragmented, but normal.
An unpunctured segment taken mid-graft exhibited inflammation which was most likely due to a cross-species immune response and coincided with lumenal thrombus, but not the vein graft itself.

Graft 9291 (Pig 161)
The arterial anastomosis showed no approximation between the native vessels and vein graft. The arterial anastomosis was free of suture line inflammation within the native carotid artery. An inflammatory response was indicated by complete isolation of the graft at the lumenal surface. A mild inflammatory response was observed within Wharton's jelly, accompanied by an insignificant amount of hematoma.
The venous anastomosis was characterized by penetration of leukocytes into the graft vein wall and a suspected cross-species immune response within the native vein as well.
Suspect opsonization products were deposited along the lumenal surface of the graft, overlying the lymphocyte reaction.
Punctures were difficult to locate and could not be positively identified. One defect suspected to be a puncture site was sealed by thrombus. Only a very slight hematoma was detected within 1 of the 4 tissue segments containing a puncture site.

Conclusions:
The decellularized, cryoprotected, and freeze-dried LifeCell umbilical vein vascular graft has been deemed suitable by Jeffrey Lawson, M.D., Ph.D. as an acute access graft for hemodialysis. The graft exhibited good handling characteristics, was easier to puncture than currently marketed synthetic PTFE grafts. In this model the grafts demonstrated a short time to hemostasis even with immediate cannulation without allowing for any healing time. Grafts with excessive coiling be a risk factor for these grafts. This study succeeded in demonstrating that these grafts are readily implantable, easily punctured and capable of providing the necessary flow rates for hemodilaysis. Further study of this vascular graft for hemodialysis access is warranted.

Appendices:
- Pre-Clinical Study Outline Form
- Pre-Clinical Protocol
- Study Director Report
- Study Observation Report
- Pathology Tracking Forms
- Study Director Report
- Anesthesia Records
- Digital Images
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  - Histology
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Appendix Q - Canine Umbilical Vein Acute Implantation Study (Summary Report)

Executive Summary:
The main objectives of this study were to place a human umbilical vein decellularized, cryopreserved, and freeze-dried by the LifeCell method in a canine model in a physiologically relevant arterio-venous shunt position (femoral artery to femoral vein) and ascertain handling characteristics including tunneling and suturing. Due to the necessity to access these grafts for hemodialysis in their eventual clinical application and due to the wide variation in umbilical vein geometry with respect to coiling, acute puncturability as well as hemostatic properties of the grafts was also assessed. In addition, patency, kinking, uniformity, and flow through the graft were evaluated both prior to and following puncture via angiography.
Two of the four grafts implanted were assessed as difficult to load into the tunneler due to the bulkiness of Wharton's jelly surrounding the vein, but all grafts slid easily through the tunneler, once loaded. Grafts were also deemed difficult to suture due to the presence of Wharton's jelly. Trimming of Wharton's jelly from the anastomotic sites on the graft to facilitate suturing, however, led to subsequent needle hole bleeding. Upon implantation, each graft had a palpable thrill beneath the abdominal wall, although the thrill in the most coiled of the grafts, which eventually occluded, decreased over time. Grafts were deemed easier to puncture than standard PTFE grafts, regardless of the extent of graft coiling. Following puncture to simulate hemodialysis access, bleeding could not be controlled with direct skin pressure for any of the four grafts. This is attributed to the lack of approximation between the graft and surrounding host tissue due to placement with a large diameter (40 Fr) tunneler. Allowing time for better integration of the graft with host tissue following implantation would likely allow for better control of bleeding. Two of the grafts became occluded following the puncture session. Of these, the lumen of the most coiled graft implanted in the study may have been compromised due to pressure caused by the large amount of blood pooling in the surgical site. The other graft may have occluded due to a combination of compression intended to control bleeding and the ensuing hypotensive state of the dog following the extravasation of a large volume of blood. A thin layer of thrombus was associated with the two patent grafts.

Objectives:
The overall goal of this study was to assess the functionality of a decellularized, cryopreserved, and freeze-dried human umbilical vein graft as a hemodialysis access graft. The criteria evaluated included handling of the graft, including suturability and subcutaneous tunneling of the graft. Ease of graft puncture with a 16G hemodialysis needle, time to hemostasis, and evidence of graft wall damage following puncture were also assessed. Finally, patency of a spiraled graft placed in a subcutaneous tunnel was examined.

Results:
A tabular display of the results collected during execution of the study can be found in Table 1. Of the four grafts implanted, three were judged difficult to suture due to

the layer of Wharton's jelly surrounding the umbilical vein. Needle hole bleeding became an issue when Wharton's jelly was trimmed from the anastomotic sites. There were challenges associated with tunneling of the graft related both to its fragility and "bulkiness" with grafts containing more Wharton's jelly being more difficult to tunnel. Once implanted, each of the four grafts, regardless of extent of coiling (Figure 1) exhibited a palpable "thrill," or high to low pressure drop typical of arterio-venous shunts. Flow through 2 of the 4 grafts was maintained for the duration of the study. Figure 2 shows a representative patent graft following explant. The remaining grafts became occluded following the hemodialysis puncture session when large volumes of pooling blood created increased pressure within the surgical cavities (Figure 3). Grafts were imaged via angiography both preceding and following the puncture sessions (Figure 4). All grafts were deemed easier to access than conventional synthetic PTFE grafts, although moderate difficulty was encountered during puncture of a particularly coiled section of the most coiled graft. Each subsequent puncture of this graft, however, was performed without difficulty.

Animal	Cord ID	Number of Coils	Length	Uniformity	Position	Ease of tunneling	Ease of suturing	Needle hole bleeding	Palpation	Flow	Ease of puncture	Time to superficial hemostasis (skin puncture site)	Hematoma following pucture	Angio-gram post-puncture	Final heparin given?	Thrombus
277	9391	5	23 cm	Uniform	Right femoral artery to right femoral vein	Slid easily through 40 Fr tunnel	Somwhat difficult	Some bleeding between sutures	Good thrill through abdominal wall but decreased over time	N/A	Easier than PTFE for 3 of 4 tries	≤5 min	Significant blood pooling in surgical site	Occluded	Yes	Clot evident near arterial anastomosis, thin layer thrombus near venous
277	9383A	0	25 cm	Uniform	Left femoral artery to left femoral vein	Slid easily through 40 Fr tunnel	Easy	Considerable; graft had been trimmed	Good thrill	N/A	Easier than PTFE	≤5 min	Significant blood pooling in surgical site – puncturing session stopped	Bleeding	Yes	Clot evident near arterial anastomosis, thin layer thrombus near venous
278	9267	2	25 cm	Uniform	Right femoral artery to right femoral vein	Difficult due to excess tissue	Difficult due to excess tissue	Some, not significant	Wonderful thrill	N/A	Easier than PTFE	5-10 min	Significant blood pooling in surgical site	Occluded	None	Very little thrombus evident throughout graft
278	9383B	2	25 cm	Uniform	Left femoral artery to left femoral vein	Difficult due to excess tissue	Difficult due to excess tissue	Some, not significant	Very good thrill	N/A	Easier than PTFE	≤5 min	Significant blood pooling in surgical site	Patent	None	Thin layer of thrombus

Table 1. Summary of all data collected during protocol execution.

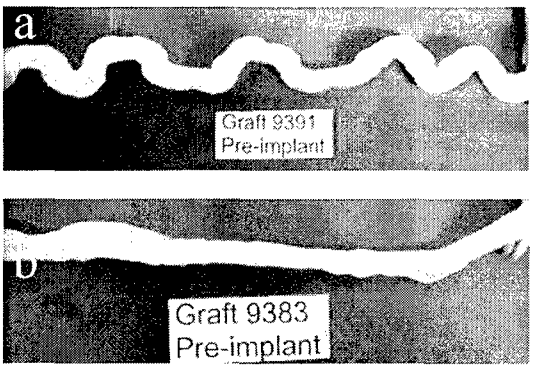

Figure 1. Representative photos of (a) very coiled and (b) less coiled grafts pre-implant.

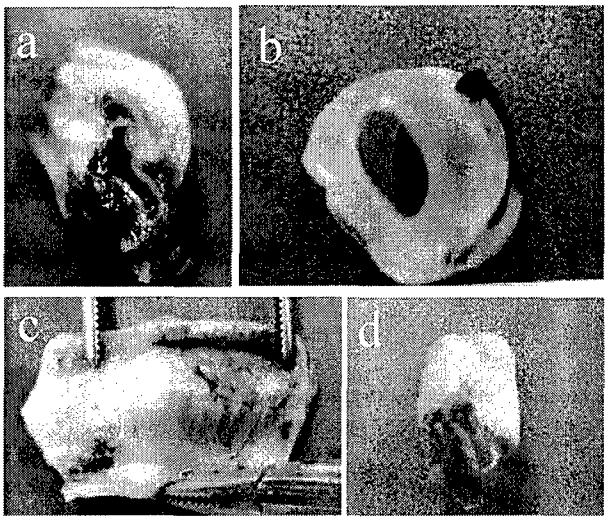

Figure 2. Explanted patent graft 9383B. (a) arterial anastomosis, (b) puncture site, (c) longitudinally opened mid-graft, (d) venous anastomosis.

Figure 3. Representative photo of graft lying in opened tunnel following puncture. Note the significant amount of clotted blood in the surgical cavity.

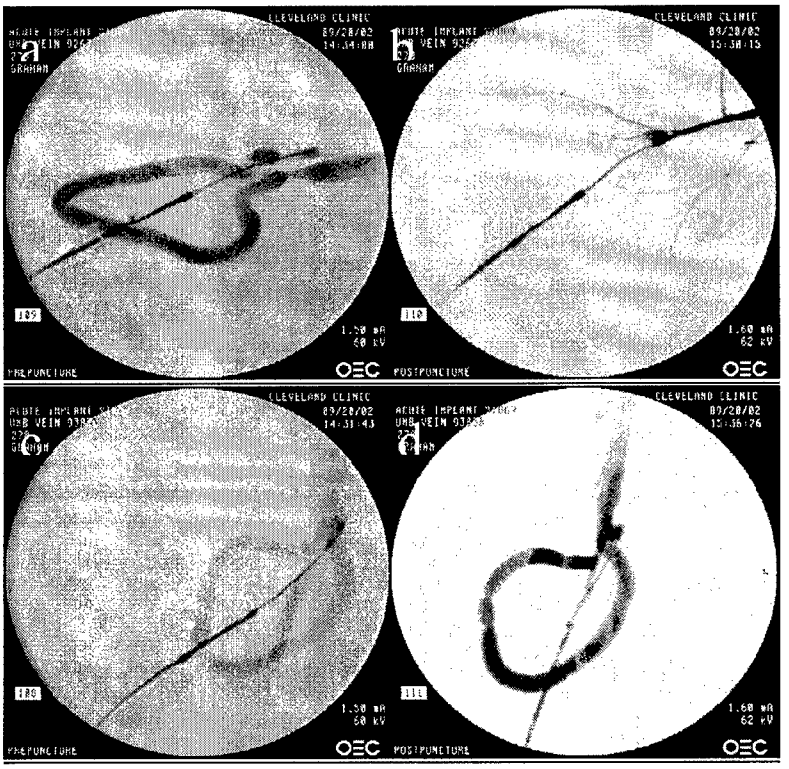

Figure 4. Representative angiograms of umbilical vein grafts in vivo. (a) Graft 9267 pre-puncture and (b) post-puncture, (c) graft 9383B pre-puncture and (d) post-puncture.

Histological observations:

Graft 9391 (Dog 277)

Both arterial and venous anastomoses showed poor approximation between the native vessels and vein graft. The arterial anastomosis showed low levels of suture line inflammation but polymorphonuclear cells and macrophages within the native carotid artery. An inflammatory response possibly due to a cross-species immune reaction was indicated by partial isolation of the graft at the lumenal surface by leukocytes. An inflammatory response was also observed within Wharton's jelly, although mostly associated with infiltrating hematoma which comprised greater than 50% of the volume of the graft in this region. A large thrombus was observed both within the native carotid artery as well as the vein graft lumen.

The suture line of the venous anastomosis had minimal to no inflammation. This section was characterized overall by isolation of the graft lumen by inflammatory cells and infiltration of leukocytes within approximately 10% of the Wharton's jelly layer. A mild inflammatory response was observed along the lumen of the native vein as well which may be due to a cross-species immune response.

Some deposited material, assumed to be fibrin, could be seen lining approximately 70-80% of the graft lumen.

Actual puncture sites were difficult to identify histologically, so areas assumed to be within 1-2 mm of three of the actual puncture sites were assessed. Of these sites, little to no hematoma was present within the Wharton's jelly layer of the graft. One

suspect puncture site was accompanied by a large hematoma within Wharton's jelly, comprising approximately 50% of the graft section.
A non-puncture site was free of hematoma but completely isolated lumenally by inflammatory cells, probably due to a cross-species immune response. Little infiltration of inflammatory cells was seen in the graft vein wall, and scattered polymorphonuclear cells could be seen within the outermost layers of Wharton's jelly.

Graft 9383A (Dog 277)
Approximation of the graft and native tissue was not evident at either the arterial or venous anastomoses as these areas of the tissue sections appeared fragmented. The arterial anastomosis was free of suture line inflammation, while the native carotid artery exhibited a very slight inflammatory response close to the anastomotic site.
An inflammatory response to the graft was indicated by isolation at the lumenal surface by inflammatory cells and may be due to a cross-species immune response. The graft appeared completely occluded by thrombus. About 40% of the volume of Wharton's jelly was composed of hematoma, which did not appear to incite much of an inflammatory response (Figure 5).
The venous anastomosis also appeared free of suture line inflammation. Inflammatory cells, which isolated the graft at the lumenal surface did not infiltrate into Wharton's jelly, which only exhibited very slight hematoma formation.
A layer of deposited material could be seen overlying the cellular inflammatory response to the graft lumen. Thrombus filling 25-30% of the graft lumen was present at this anastomosis as well.
Suspected punctures were accompanied by very slight hematoma within Wharton's jelly. One puncture site that was positively identified appeared to be sealed by fibrin and a modest platelet plug (Figure 6) in the section evaluated. A non-puncture site was characterized by isolation of the graft lumen by inflammatory cells, but no inflammation or hematoma within Wharton's jelly. An occlusive thrombus was seen in this section as well.

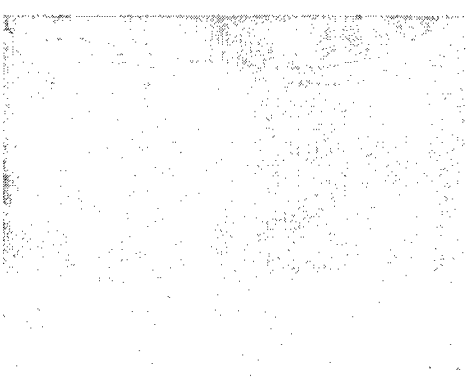

Figure 5. Wharton's jelly layer of graft 9383A, infiltrated by hematoma.

Figure 6. Punctured site of graft 9383A with accompanying fibrin plug.

Graft 9267 (Dog 278)
The arterial anastomosis showed good approximation between the native vessel and vein graft, with some inflammation at the suture line but none within the native carotid artery. An inflammatory response was indicated by complete isolation of the graft at the lumenal surface by lymphocytes, which was overlaid by proteinaceous deposit. It is possible that this inflammatory response is due to a cross-species immune response. Virtually no thrombus was seen in this section of the graft. The vein wall itself was partially invaded by some inflammatory cells in scattered areas, but no cells were observed to infiltrate Wharton's jelly. A trivial amount of hematoma within Wharton's jelly was observed nearest the outer most layers of the graft.
The venous anastomosis exhibited an inflammatory response isolating the graft lumen as well as moderate infiltration of inflammatory cells into the vein wall itself, overlaid with proteinaceous deposits and thrombus. A most likely occlusive thrombus was also seen at the anastomotic site. No inflammatory cells were found within Wharton's jelly, although a slight hematoma was found within this layer of the graft. A moderate inflammatory response could be seen within the native jugular vein, accompanied by tissue damage and lumenal fragmentation.
Three actual puncture sites were accompanied by moderate but localized hematoma within the Wharton's jelly layer of the graft. The actual fourth site could not be positively identified, but slight hematoma within Wharton's jelly was observed in the general vicinity.
An unpunctured segment exhibited moderate hematoma within Wharton's jelly and an inflammatory response as well as non-occlusive thrombus surrounding the graft lumen.

Graft 9383B (Dog 278)
The arterial anastomosis showed good approximation between the native vessel and vein graft and appeared free of suture line inflammation, while the native carotid artery itself exhibited a slight inflammatory response. Slight hematoma, but no inflammatory response, was observed in Wharton's jelly. The graft was isolated at the lumenal surface by inflammatory cells, although none penetrated the vein wall. This inflammation may be due to a cross-species immune response. No thrombus was detected within the lumen.
The venous anastomosis showed poor approximation between the vein graft and native jugular vein, and the suture line could not be positively identified. Slight inflammation was evident in the native vessel close to the anastomotic site. The graft wall itself was isolated by inflammatory cells which did not penetrate the vein wall or surrounding Wharton's jelly. Slight hematoma was observed in Wharton's jelly. Fibrin deposits, but no clear thrombus was evident in the graft lumen.
Two puncture sites were found to be lined, but not completely sealed, with fibrin and did not have accompanying localized hematoma. The remaining two punctured sites were not positively identified, although the surrounding Wharton's jelly exhibited slight and moderate hematoma, respectively.

A non-puncture site had lumenal isolation by inflammatory cells. Moderate hematoma was seen within Wharton's jelly adjacent to the vein wall in approximately 1/3 of the lumenal circumference and accompanied by inflammatory cells.

Conclusions:
Time may be needed to allow the graft to heal in and become more closely approximated to host tissue prior to attempting angioaccess. Grafts which became occluded in this study were likely compromised by excessive blood extravasation that will not occur clinically when access is made after appropriate healing time. The decellularized, cryoprotected, and freeze-dried LifeCell umbilical vein vascular graft was significantly easier to access for hemodialysis than currently marketed synthetic PTFE grafts. While the graft's handling characteristics were not optimal this study succeeded in demonstrating that these grafts are readily implantable. Further study of this vascular graft for hemodialysis access is warranted.

Appendices:
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Appendix R - In Vivo Assessment of Umbilical Vein Grafts in a Porcine Model – Feasibility Study (Summary Report)

Executive Summary:
The main objectives of this study were to place two human umbilical vein decellularized, cryopreserved, and freeze-dried by the LifeCell method in a porcine model in a physiologically relevant arterio-venous shunt position (carotid artery to jugular vein) and ascertain appropriate suture material as well as anti-platelet therapy to be used. The issue of tunneling these unique biologic grafts was also to be addressed. In addition, flow through the grafts, palpation characteristics, and patency were evaluated following 12 days of implantation. Additional data pertaining to hemostasis following anastomosis and histological assessment of explanted tissue were also collected.
Two different types of suture were used in the anastomoses of each graft. Non-resorbable 6-0 Prolene suture was used at one anastomosis and compared to resorbable 4-0 PDS suture at the other anastomosis. No suture hole bleeding occurred at either anastomoses of either graft, despite the larger needle and suture associated with 4-0 PDS. Each graft had a palpable thrill beneath the subcutaneous tissue of the neck which could be felt throughout the duration of the 12 day study. Both grafts remained 100% patent as assessed by angiography and gross evaluation and were found to support flows of between 300 and 670 mL/min just prior to explant. Following explant of the grafts *en bloc*, both grafts were found by Evans blue dye exclusion to exhibit patchy endothelialization in central regions of the graft. No thrombus was associated with the lumen of either graft. However, large areas of hematoma within the Wharton's jelly layer did appear to be associated with lumenal defects in one of the grafts.
The current anti-platelet therapy regime, consisting of daily aspirin and Plavix and perioperative heparin, appeared to be adequate in eliminating the risk of acute graft failure due to thrombus in this model and would most likely be suitable for a longer term study involving graft puncture.
Severe inflammation due to a cross-species immune response masked any differences in inflammation elicited by either suture type used. The severity of this response compromises the value of further long-term implant studies with this cross-species model.

Objectives:
The overall goal of this study was to assess the functionality of a decellularized, cryopreserved, and freeze-dried human umbilical vein graft as a short-term arterio-venous shunt. The criteria evaluated included subcutaneous tunneling of the graft, selection of appropriate suture, and selection of adequate anti-platelet therapy, allowing for maintained graft patency as assessed by flow, angiogram, and palpation. In addition to these criteria, secondary objectives related to host/graft interaction (histology to assess possible cross-species immune response and Evans blue dye exclusion) were evaluated.

Results:
A tabular display of the data collected during execution of the study can be found in Table 1. Both grafts were judged to suture well without needle hole bleeding despite the large needle and suture used with PDS. Little challenge was presented in tunneling of

the grafts, as each had a minimal degree of coiling (Figure 1). Once implanted, each graft exhibited a palpable "thrill," or high to low pressure drop typical of arterio-venous shunts which was detectable for the duration of the study. Flow through both grafts was maintained at greater than 300 mL/min for the duration of the study. See Figure 2 for angiograms of the grafts immediately following implant and after 12 days. Both grafts remained 100% patent and free of thrombus through explant despite hematoma formation within the Wharton's jelly layer of one graft. Areas of hematoma in this graft appeared to be associated with lumenal defects in the graft (Figure 3). Despite hematoma formation and a severe immune response related to the cross-species implant (see histology section), patchy endothelialization was observed by Evans blue dye exclusion throughout the length of both grafts (Figure 4). No lumenal defects were detected in the graft free of hematoma.

Appendix R - In Vivo Assessment of Umbilical Vein Grafts in a Porcine Model – Feasibility Study (Summary Report)

Animal	Cord ID	Number of Coils	Length	Position	Suture	Needle hole bleeding	Palpation	Flow	Angio-gram at 12 days	Evans Blue exclusion	Hematoma	Defects in graft wall	Final heparin given?	Thrombus
24	9550	0	14 cm	Left carotid to right external jugular	Arterial: 4-0 PDS Venous: 6-0 Prolene	Arterial: None Venous: None	Good thrill evident at 4, 8, and 12 days	300+ mL/min at 12 days	100% patent	Patchy, throughout length	None	None	yes	None
25	9378	1	18 cm	Left carotid to right external jugular	Arterial: 6-0 Prolene Venous: 4-0 PDS	Arterial: None Venous: None	Good thrill evident at 4, 8, and 12 days	670 mL/min at 12 days	100% patent	Patchy, throughout length	In Wharton's jelly	Several	yes	None

Table 1. Summary of data collected during protocol execution.

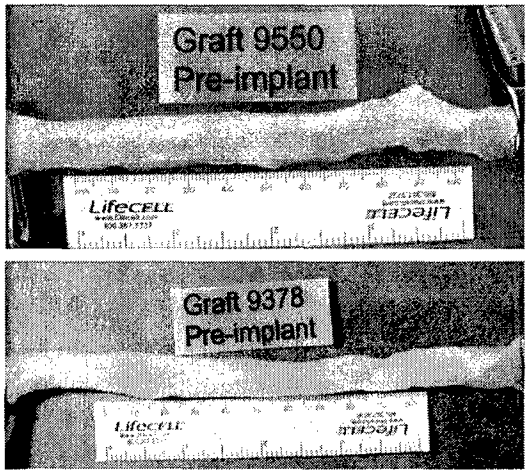

Figure 1. Photos of rehydrated grafts pre-implant.

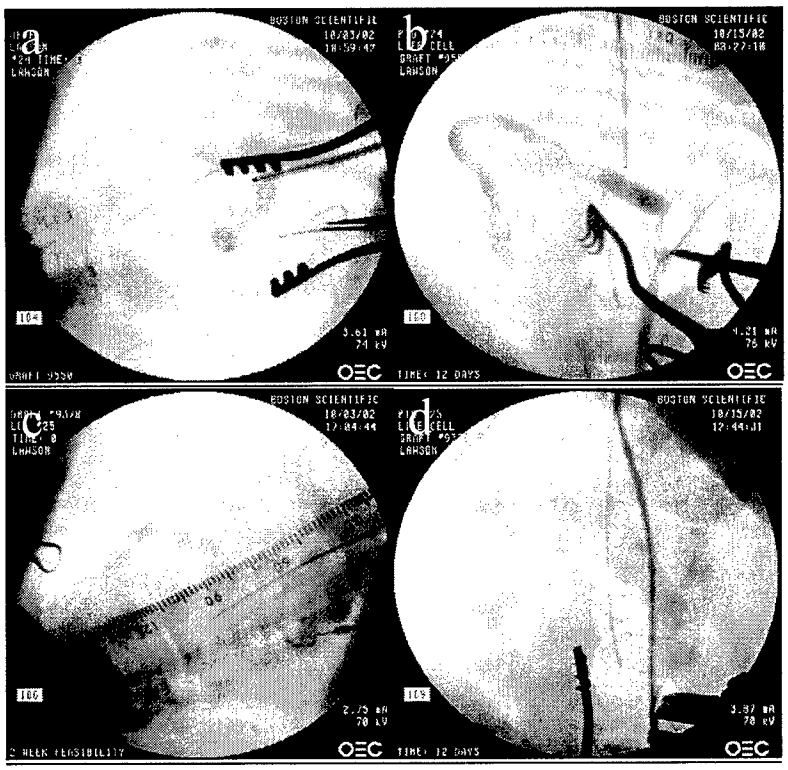

Figure 2. Angiograms of umbilical vein grafts in vivo (a) graft 9550 at implant and (b) after 12 days; (c) graft 9378 at implant and (d) after 12 days.

Figure 3. Graft lumen with underlying hematoma within Wharton's jelly.

Figure 4. Graft lumen exhibiting patchy endothelialization by Evans blue dye exclusion.

Histological observations:
Graft 9550 (Pig 24)
The arterial anastomosis showed some inflammation at the PDS suture line (Figure 5) and a lack of approximation of the native and graft tissues. Large populations of leukocytes could be found within the native carotid artery, with less migrating into the Wharton's jelly surrounding the vein graft. The vein wall itself had a slight inflammatory response at the lumen with very little repopulation by fibroblast-like cells.

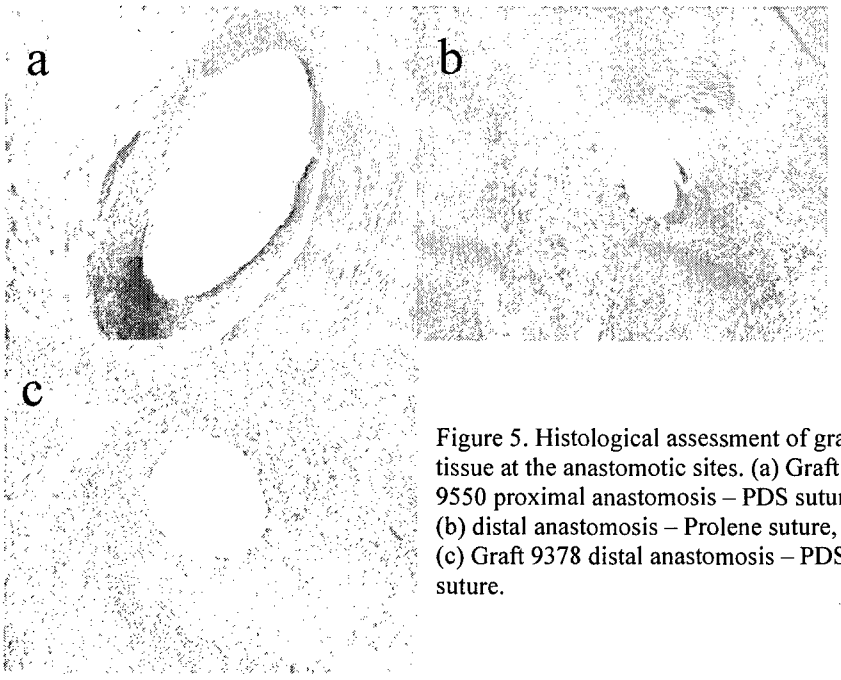

Figure 5. Histological assessment of graft tissue at the anastomotic sites. (a) Graft 9550 proximal anastomosis – PDS suture, (b) distal anastomosis – Prolene suture, and (c) Graft 9378 distal anastomosis – PDS suture.

In both the proximal and mid-portions of the graft, an intense inflammatory response, due to a cross-species immune reaction, was apparent within the native tissue along the graft/native tissue junction and a lesser reaction along portions of the lumenal surface (Figure 6). Here, approximation of the graft and native tissue appeared to be very good. The graft appeared to be repopulated along a gradient within Wharton's jelly, with the least cells infiltrating the graft tissue toward the

lumen in some regions. Other areas, however, were heavily repopulated throughout Wharton's jelly. Repopulation of the mid-portion of the graft was very organized in the circumferential direction where surrounding native tissue was less organized. A protein deposit was found overlying the lumen in some regions of the mid-portion of the graft. Some scattered areas of the vein wall were also repopulated, with endothelialization occurring in some regions of the lumen in both the proximal region and mid-portion of the graft (Figure 7).

Figure 6. Host tissue inflammatory response along graft/native tissue junction.

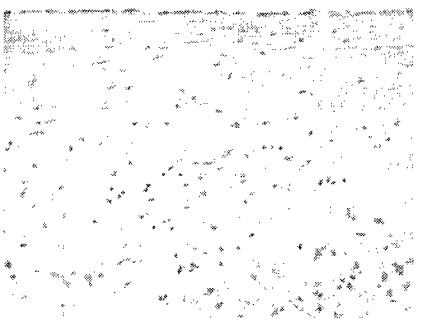

Figure 7. Histological evidence of graft lumenal re-endothelialization.

The distal region of the graft exhibited vein wall damage in some areas, repopulated by fibroblastic cells in other areas, or infiltrated with inflamatory cells. A deposit of fibrous material was found along approximately 20% of the lumenal surface. As in the proximal and mid-portions of the graft, an intense cross-species immune reaction, was found with the distal native tissue. Repopulation of Wharton's jelly was intense and circumferentially organized.

The native jugular vein at the venous anastomosis looked normal and was approximated nicely to the graft. The Prolene suture line looked somewhat inflamed (Figure 5), but not any more so than the surrounding tissue. Areas of the vein wall itself appeared thin or damaged. Vascularization was found in regions very close to the vein wall which could either indicate that little Wharton's jelly existed in this area to begin with, Wharton's jelly had been revascularized, or that the material which had surrounded the vein wall had been degraded.

Graft 9378 (Pig 25)

Very little native tissue was explanted along with this graft because of a lack of adhesion. This made assessment of tissue approximation limited.

The Prolene suture line at the arterial anastomosis was not evident. Tissues were approximated nicely, and a distinction between graft and native tissue was difficult to draw. An intense inflammatory response was seen within the native carotid artery, but very little within the vein graft wall or Wharton's jelly. Wharton's jelly was nicely repopulated, unlike the vein wall.

In the proximal portion of the graft, the vein wall appeared thin and damaged in some areas, with regions of separation from Wharton's jelly. Repopulation and inflammatory responses varied, with approximately 50% repopulation of Wharton's

jelly and 50% of the vein lumen infiltrated by a severe inflammatory response. Nearly one-third to one-half of Wharton's jelly was occupied by hematoma (Figure 8).

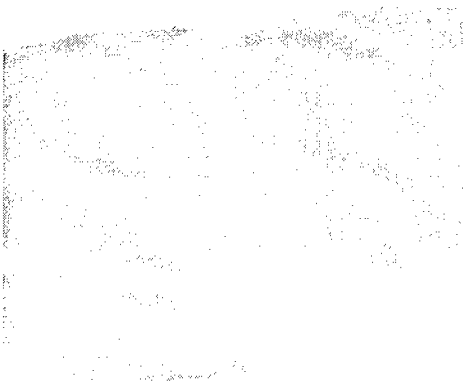

Figure 8. Hematoma within Wharton's jelly layer of graft wall.

Mid-graft, even more hematoma was evident (50-75%) within Wharton's jelly, and an intense cross-species immune reaction, was evident at the vein wall/Wharton's jelly junction. The vein wall itself was very thin in some areas, with little or no repopulation or inflammation. An area of re-endothelialization was clearly visible, although it comprised a small percentage of the graft lumen (~5%).

Distally, approximately one-third of the graft was permeated by hematoma with an accompanying inflammatory response within the vein wall. Although the majority of Wharton's jelly was not repopulated, some scattered endothelial cells could be seen at the lumenal surface.

Good approximation of the graft and host tissue was evident at the venous anastomosis, where the PDS suture line was inflamed (Figure 5), but not more so than the surrounding tissue. Although localized regions of hypercellularization and a small area of hematoma were evident, overall, the native jugular vein appeared normal. The graft vein wall appeared extremely thin and damaged in several areas, exhibiting infiltration by both scattered fibroblastic cells and leukocytes. The majority of Wharton's jelly was not well repopulated. An intense cross-species immune response was evident in the most distal portion of the graft.

Conclusions:

No conclusions could be drawn regarding tissue response to different sutures used at the anastomoses, as any inflammatory response was masked by the cross-species immune response (see study director report and attached pathology report). The anti-platelet regimen used on the animal subjects appeared to be adequate in preventing anastomotic thrombosis, as no thrombus was evident within the grafts or adjacent native tissue. Defects in one graft, were associated with underlying hematoma within Wharton's jelly and attributed to either pre-existing cracks in the lumen or the severity of the immune response causing rapid degradation of the tissue. Cracks found on the lumen of other non-implanted vessels are caused by non-optimal freeze drying. The grafts exhibited a low level of endothelialization despite the severe immune response.

The decellularized, cryoprotected, and freeze-dried LifeCell umbilical vein vascular graft functions well as a short-term arterio-venous shunt, as assessed by palpation, patency, and flow over a 12-day implantation period. The severity of the cross-species immune response compromises the value of further long-term implant studies with this cross-species model.

Appendices:

- Pre-Clinical Study Outline Form
- Pre-Clinical Protocol
- Study Director Report
- Study Observation Report
- Pathology Tracking Forms
- Anesthesia Records
- Digital Images
    - Peri-operative
    - Post-explant
    - Angiography
    - Histology
- Histology Runsheet

Appendix S - Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

Objectives

The human umbilical vein graft is intended to function as an arteriovenous (AV) access graft in hemodialysis patients. The only large animal study performed to date was a porcine model[1] where the results included severe inflammatory response attributed to cross-species incompatibility. Cross-species immune response can play a role in chronic graft failure so no further chronic studies were pursued. Recent data in a hernia repair model using AlloDerm in the African Green Monkey indicate that there may be close enough homology with humans for processed human tissue to avoid significant immune reactivity.

The objective of this study was to evaluate the suitability of the African Green Monkey for animal implantation with the Human Umbilical Vein Graft for the purpose of evaluating the safety of that graft.

Using the longest graft segment that can be achieved without compromising the graft or animal, the Human Umbilical Vein Grafts were implanted in large male vervets (one per animal) as an AV shunt from the Iliac/femoral artery to the femoral vein. Needle access was planned as a possible endpoint to demonstrate the potential for a chronic multi-stick model. Grafts were implanted for 2 and 6 weeks, three for each time point, with 3 additional grafts included with an unplanned duration. Synthetic control grafts were also implanted (one graft for each time point). Serum was obtained prior to and during the study to evaluate the immune response of the animal to the graft.

Results

A. Study observations

Twelve male African Green monkeys (Vervet, *Cercopithecus aethiops*) were identified by number and randomly assigned to either study or control group. All animals were screened for general health and quarantined for 2 weeks before study entry. All monkeys were housed in individual cages and allowed free access to water, fruits, and monkey food. At the time of surgery the animal weights ranged from 4.45 - 5.62 kg.

Umbilical Vein Grafts (UVG) were transported to the animal testing facility on dry ice. Temperature of the grafts was maintained below -30°C during transport. After transport the grafts were maintained on dry ice until within 4 hours of implantation. The standard thaw and rehydration procedure described in the DFU was used before implantation.

- Test Articles
  Human Umbilical Vein Grafts that were processed per LOP09.01.058 and maintained at -65°C to -90°C until shipment on dry ice to the test site. Test graft articles consisted of approximately 6-8 cm long pieces of whole grafts. The test grafts used in this study were lot numbers U00023, U00033, U00037B.

- Control Articles
  Segments of 6-8 cm × 6 mm diameter ribbed ePTFE implanted in a similar manner to the test article in 3 additional animals (one for each time-point).

[1] LCP-2002-10-02 – performed in 2002.

An anatomical comparison of the upper leg / groin area to the neck of the animals indicated that the former was a more likely candidate for successful grafting. Monkeys were anesthetized per the protocol and the iliac artery and femoral vein were exposed by sharp and blunt dissection. A segment (6-8cm) of umbilical vein graft or synthetic (ribbed) ePTFE graft (6mm diameter) was anastomosed to the iliac artery and femoral vein creating an AV shunt. Non-absorbable 6-0 or 7-0 sutures were used. The loop of graft was placed subcutaneously to allow non-invasive evaluation of flow and cannulation. Appropriate antibiotics were administered for 3 days after surgery. No antiplatelet or anticoagulant agents were given during or following surgery.

B. Implantability

Nine UVG segments from three different UVG and three synthetic grafts were successfully implanted - one in each of 12 animals – by Dr. Shiji Qi, over a three day period. One control graft and three UVGs from a single lot were implanted each day. See Table 1 for additional implantation details. Patency was verified by palpation and/or Doppler flow probe (Appendix A – Implant Data Forms).

The grafts were evaluated for patency immediately after completion of implantation and then again after closure by palpation of the graft. An available Doppler flow probe (Smart Doppler ES-1000SPM, Koven Technologies, St. Louis MO) was used to evaluate its ability to measure flow velocities in the implanted grafts. While there is no certainty regarding the quantitative ability of the probe, it was found to qualitatively verify patency of the grafts.

There were no cases of suture pull-out or significant bleeding during implantation. In one case the UVG twisted when arterial flow was initiated in the graft causing a tear in the femoral vein tissue near the anastomosis that was successfully repaired. The synthetic grafts had minor bleeding at the suture holes that was easily stopped with pressure. Despite four grafts with 2 or more turns or coils (#O5481, #O5486, #O5496, #O5493) and a graft loop with a radius of approximately 0.5 cm for the UVG, there were no instances of kinking. The ribbing on the synthetic graft provided protection from kinking by limiting the radius of the graft loop. Photographic records of the implanted grafts *in situ* and after surgical closure are included as Appendix B.

Table 1: General Imlant Data (Appendix A – Implant Data Forms)

Animal Number	Implant Number / Type	Animal Weight (kg)	Date of implant	Mean Flow (cm/s)	Procedure time (min)	# coils
O5480	U00033	4.87	4/20/2004	4.5	67	0
O5481	U00023	4.51	4/19/2004	2.4	80	2
O5485	U00037B	4.76	4/21/2004	3.2	50	1
O5486	U00023	4.76	4/19/2004	4.1	90	3
O5496	U00037B	5.19	4/21/2004	5	50	2
O5499	ePTFE control graft	5.34	4/19/2004	2.8	110	N/A
O5109	U00037B	4.79	4/21/2004	3.5	not recorded	1
O5484	U00023	5.59	4/19/2004	5	60	not recorded

O5460	ePTFE control graft	5.62	4/20/2004	2	76	N/A
O5494	U00033	4.63	4/20/2004	5.1	65	1.5
O5483	ePTFE control graft	4.98	4/21/2004	3.5	85	N/A
O5493	U00033	4.45	4/20/2004	11.2	60	2

C. Deviations

Storage temperature: The protocol calls for storage temperature of the grafts to be maintained at -65°C to -90°C. During shipment of the grafts on dry ice the temperature probes within the shipper briefly reached as high as -30°C[2]. The actual temperature of the grafts at that time is unknown. This deviation in storage is not expected to influence the results of this study. Sections of the grafts obtained at the time of implantation (and after shipment) were evaluated histologically and were not found to be any different than pre-shipment sections.

Control grafts: The protocol called for an 8 mm diameter crimped Bionit Dacron graft (C.R. Bard, Inc., Billerica MA). A 6mm ePTFE graft was used instead. This graft material is the standard of care in the hemodialysis industry and therefore a more appropriate control for the UVG in this study. The deviation does not affect the results of the study.

Early sacrifices: One umbilical vein graft was disturbed by the monkey after five weeks but clotted off before resulting in death of the animal and two other grafts (one control and one UVG) had been repeatedly exposed by the monkey and secondary and tertiary closure of the surgical site was not healing. In these cases the monkeys were sacrificed at five weeks rather than six weeks to avoid the risk of losing additional animals from the study. The deviation impacts the data as it allows for an evaluation of the chronic response of five weeks duration instead of six weeks.

Data completion: The protocol calls for evaluation of cytokine levels and complement activation if there is a sign of inflammation. The immunological assessment provided by the evaluation of antibody levels and immunohistochemistry were later considered to be sufficient information to assess the model.

D. Adverse Events

All adverse events have been defined and reported in an adverse event report from the Staff Veterinarian at the Behavioral Sciences Foundation (Appendix C). Adverse events in this study included ischemia, graft rupture and animal death. A degree of ischemia of the lower extremity was caused by diversion of the arterial flow. The ischemia was noted as a qualitative temperature difference between implanted and contralateral limbs and in some cases temporary immobility of the foot on the implanted limb. Three animals died during the course of the experiment. The cause of death in those cases was linked to exposure and disruption of the graft by the animal. In another case the graft was torn open by the animal but clotted off by itself. This event precipitated an early sacrifice of the animal at five weeks. Full description of these events and photographic record are included in the Adverse Event report (Appendix C). Another animal intended for sacrifice at six weeks was sacrificed at five weeks due to uncertainty surrounding the likelihood of the animal disrupting the graft.

[2] experiment 238-03 "International shipping package containing vein grafts U00023, U00033, and U00037B"

E. Patency

Patency was evaluated on a weekly basis non-invasively by palpation of the graft and by Doppler ultrasound. After sacrifice, the grafts were explanted and flushed with normal saline or RPMI solution to remove non-adherent blood. This procedure also served to further verify patency. All grafts in planned sacrifices, UVG and synthetic, remained patent (Table 2).

Explanted grafts were sent to LifeCell for dissection and preparation for histological sectioning and staining. The photographic record of the explant dissection demonstrates little adhesion of blood or thrombus to the lumen of the UVG (Appendix D).

Table 2: Explant duration, patency record and time to hemostasis after cannulation.

Animal #	Graft	Duration (days)	Duration Group	Patent at Explant	Time to hemostasis (min)
05499	ePTFE	16	2 week	Yes	not provided
05486	U00023	16	2 week	Yes	5
05480	U00033	15	2 week	Yes	>5
05496	U00037B	14	2 week	Yes	3
05460	ePTFE	36	5-6 week	Yes	2
05483	ePTFE	42	5-6 week	Yes	3-4
05484	U00023	36	5-6 week	Yes	3-4
05494	U00033	36	5-6 week	Yes	3
05493	U00033	43	5-6 week	Yes	5-6
05481	U00023	14			
05485	U00037B	10	No data colleted due to graft disturbance and subsequent animal death		
05109	U00037B	17			

F. Puncturability / Hemostasis

Prior to explantation the grafts were cannulated using a 16g needle as prescribed in the protocol addendum. Hemostasis was achieved using external pressure in <5 minutes in four of the 6 cannulated grafts with the other two requiring >5minutes. One synthetic graft (#O5499) was reported to have uncontrolled bleeding (Table 2). In two cases high pressure was reported during the cannulation procedure indicating a restriction in venous outflow likely the result of the diameter mismatch from the graft (6-8mm) to the vein (2-3mm).

G. Animal Observations – photographic record

H. Explant Record – gross evaluation

The graft were photographed at explant and shipped to LifeCell Corporation in RPMI on ice. Upon receipt of the tissue the grafts were photographed, dissected and sectioned into the following pieces:

- Arterial anastamosis was bisected ½ for sucrose cryoprotectant (OCT) and ½ for formalin storage.
- Serial sections (2 cm) of the graft were made starting from the arterial side after the anastomosis. They were alternately stored in OCT and formalin and numbered from one and increasing from arterial end to venous end.
- Venous anastomosis was bisected ½ for OCT and ½ for formalin storage.

Photographs were taken of the lumen of the graft and the anastomoses (Appendix D).

I. Biological Response

Histological evaluation

An independent histopathological review of hemotoxylin and eosin (H&E) stained sections was performed on all graft sections for two different groups of explants: 1.) those explanted at 14 days (including all grafts explanted from non-surviving animals) and, 2.) those explanted at 5-6 week durations. The final summary report is attached as Appendix E. This report indicates that at early time points (14 day) there was a significant mixed inflammatory response to the graft consisting of polymorphonuclear cells (primarily neutrophils) and macrophages (with a minor component of giant cells). At 5-6 weeks, a mixed inflammatory response was still present but notably reduced. Also, by 5-6 weeks, there was evidence of healing (resolution of the inflammation) with concomitant new matrix (fibrous connective tissue) formation. There was an absence of aneurysm, tissue dissection, or significant thrombus formation. The early inflammatory response and histology are typical for biological implants.

Immunologic response

The cellular response described by H&E pathology was evaluated further using immnohistochemistry. The sections were stained for the presence of macrophages (CD68), T cells (CD3) and B cells (CD20). There was extensive infiltration of macrophages at 14 days especially around the sutures and within the graft as well. This is an expected response to a foreign (not yet integrated) material and is generally understood as the necessary precursor to an infiltration of host cells in a subsequent healing response. The degree of macrophage infiltration was milder and more localized at five and six weeks indicating an acceptance of the tissue. In contrast, macrophages were found in all sections in the host tissue surrounding the synthetic grafts. T cells were found in the host tissue surrounding the graft. However, occasional T cells were found in the wall of vein grafts, especially at the early time points. The explants from two monkeys at five and 6 weeks (#05494 & #05493) showed minimal infiltration of T cells. B cells were generally not observed in most of the sections. In some cases B cells were found in the monkey tissue surrounding the grafts.

Serum was obtained before implantation and at regular intervals following implantation. The serum was stored refrigerated and shipped frozen to LifeCell Corporation after completion of the study. The serum was tested to determine if the animals raised antibodies to the implanted grafts or equivalently processed grafts from different donors. Overall, vascular grafts induced an IgG response in monkeys 14 days post-implant although IgM antibodies remained similar to the pre-implant levels. It is unlikely that monkeys respond to donor specific antigen epitopes since less binding to donor-specific grafts than to generic grafts was detected (Appendix F - Immunology Summary Report). This data indicates that the tissue has been effectively decellularized as defined by the removal of antigenic capacity.

Appendix S - Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

Model Assessment

There are a number of aspects of this model that require further development and/or mitigation and may affect the ability to use this model for chronic evaluation of the graft.

- In 4 of 12 cases the monkeys disturbed their grafts leading to death in three of those cases. For this model to be successful, modifications need to be implemented that can reduce the occurrence of graft disturbance.
- The mismatch in graft diameter compared with the vessels of the monkey may increase the probability of thrombosis in this model.
- The significant length of graft positioned subcutaneously in the groin area may be prone to bending, kinking and external pressures during the course of daily activity, particularly as the monkeys tend to spend a lot of time in a crouched position.
- The 6-8cm length of graft placed in such a small space necessitates a graft loop with a very small diameter (~1cm) that may create hemodynamics that initiate thrombosis.
- Since the graft segments were relatively long, it was necessary to place the grafts perpendicular to the anastomoses while a 30 degree angle of anastomosis would have been more optimal for smooth blood flow to avoid turbulence that can activate platelets and thrombotic events.

Possible improvements for the model include:

- Implementation of safeguards to prevent animals from disturbing the grafts. The most significant change to incorporate is modification to the method of closure. Closure of the surgical site should be completed in layers including subcuticular and intradermal sutures and only interrupted stitches should be used.
- A less aggressive model might be possible using a much shorter segment of the graft in a straight (not looped) configuration with angled anastomoses. The utility of the model for assessing kinking, cannulation, and repopulation and endothelialization would be limited. With the limited size of the monkey thigh, it is not clear that this model is feasible.

Conclusions

This study was designed to use the African green monkey as a model to study the human umbilical vein graft. This study demonstrates that the umbilical vein graft (UVG) can be implanted as an arteriovenous shunt from the iliac artery to the femoral vein in this primate and remain patent for as long as six weeks. The data show that there is a humoral immune response to the graft in this model. However, despite this response, no mural degeneration or aneurysms in the graft were noted within the first six weeks. An initial inflammatory reaction to the graft was shown to resolve over time giving way to a healing response and the initiation of new tissue growth. Taken together these results indicate that although this is a xenogeneic model (Human to Monkey), it may provide a means of obtaining information about the expected function, hemocompatability and healing response of the UVG for as long as six weeks *in vivo*. This study also demonstrated that the graft can be cannulated in the chosen position and that hemostasis after cannulation of the UVG compares favorably to ePTFE.